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CHARACTERIZATION OF TISSUE METABOLISM OF TRANSPLANTED MOUSE MELANOMAS BY HIGH OXIDATIVE RESPONSE TO PARAPHENYLENEDIAMINE[1,2]

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INTRODUCTION

Various papers and discussions at the recent conference on pigment-cell growth[3] have emphasized the slow growth and prolonged killing time of the three transplantable melanomas available in mice, the Harding-Passey and Cloudman S91 pigmented melanomas and the Algire S91A amelanotic melanoma. Only the Cloudman S91 melanoma has metastasized regularly, primarily to the lungs, but also to the kidneys, adrenals, lymph nodes, orbit, and liver. Particularly noteworthy are certain biologic results obtained with these three melanomas when they are transplanted into transparent chambers in skin flaps of mice. In contrast with highly malignant sarcomas and mammary-gland carcinomas that have been similarly transplanted, the melanoma cells migrate slowly and elicit new capillaries only after a prolonged latent period, with little leukocytic accumulation about the implant; vascular dilatation in the surrounding tissues is minimal; vascular levels within the melanomas do not exceed that of circumambient subcutaneous tissue; and the capillaries formed are small in diameter, like those of normal striated muscle, and show considerable differentiation into arterioles and venules (*1*).

It was of interest, therefore, to examine tissue slices of these mouse melanomas from a metabolic standpoint, to see whether there might be some biochemical basis for the observed, biologically distinctive behavior. A common metabolic feature or features might be found that would differentiate these tumors, as a class, from other malignant tumors, apart from any differences that might exist between the three melanomas themselves, particularly between the melanotic S91 strain and the amelanotic S91A strain derived therefrom. The values given by Crabtree (*2*) for four specimens of mouse melanoma are about the only data reported heretofore on the metabolism of melanoma tissue slices as distinguished from tissue homogenates thereof, with which this paper is not directly concerned experimentally.

RESULTS

Tables 1 and 2 present a summary of experimental data obtained to date with six or more tumors taken from each melanoma strain and involving a total of several hundred individual Warburg vessel determinations carried out by methods described in detail elsewhere (*3–6*). The Harding-Passey melanoma was carried mainly in C3H mice, the Cloudman S91 melanoma in dba mice, and the Algire S91A amelanotic melanoma in C mice. For comparison, determinations were made on the Earle L sarcoma and the Barrett C3HBA adenocarcinoma, both carried in C3H mice. Metabolic measurements of individual spontaneous adenocarcinomas in C3H mice gave values indistinguishable from those given by the transplanted C3HBA strain.

When judged by the metabolic criteria listed in table 1, the data on all five tumor strains fall within the range of Q values shown by malignant tumors generally (*7–9*), as summarized in the last column of the table. Within these limits, there is some tendency for all the experimental values, anaerobic glycolysis, aerobic glycolysis, oxygen consumption, and carbon dioxide production, to increase in the order of tumors as listed. The two melanotic melanomas show the lowest values. This might be accounted for, in part, if the melanin constituted a considerable part of the dry matter and was metabolically inert. There is little gradation between the tumors with respect to the derived ratios: respiratory quotient, extent quotient, fermentation excess, and absolute Pasteur effect. The percent Pasteur effect and the

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[2] The bulk of this material was presented at the conference on the Biology of Normal and Atypical Pigment Cell Growth held at the New York Academy of Sciences, November 15 and 16, 1946.

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Meyerhof oxidation quotent, however, show decreases in the order of tumors listed. All gradations, it should be emphasized, are nevertheless within the usual malignant-tumor limits, as established in the work of Warburg, Dickens, and many others (*7-10*), due consideration being given to use of estimated initial rather than final dry weights as a basis for comparative calculation.

TABLE 1.—*Standard metabolism of tissue slices of transplanted mouse melanomas and other malignant tumors in Warburg-Okamoto-Ringer-glucose-bicarbonate medium* [1]

Tumor	Harding-Passey melanotic melanoma	Cloudman S91 melanotic melanoma	Algire S91A amelanotic melanoma	Earle L sarcoma	Barrett C3HBA adenocarcinoma	Malignant tumors in general (approximate range)
Experimental:						
$Q^{N_2}{}_A$, anaerobic glycolysis (acid production in 5 percent CO_2/N_2)	11.0	11.8	14.8	12.0	17.6	8–25
$Q^{O_2}{}_A$, aerobic glycolysis (acid production in 5 percent CO_2/O_2)	2.1	3.0	5.2	5.6	10.4	2–15
Q_{O_2}, oxygen consumption	4.6	5.0	7.2	7.7	9.4	3–10
Q_{CO_2}, carbon dioxide production	4.1	3.8	6.0	6.4	8.6	2–9
Derived ratios.						
$3Q^{N_2}{}_A/Q_{O_2}$, extent quotient	7.2	7.1	6.2	4.7	5.6	4–20
$U=Q^{N_2}{}_A-2Q_{O_2}$, fermentation excess	1.8	1.8	.4	−4.3	−1.2	−5–20
$Q^{N_2}{}_A-Q^{O_2}{}_A$, absolute Pasteur effect	8.9	8.8	9.6	6.4	7.2	6–15
$100(Q^{N_2}{}_A-Q^{O_2}{}_A)/Q^{N_2}{}_A$, percent Pasteur effect (percent inhibition of $Q^{N_2}{}_A$ by O_2)	81	74	65	53	41	40–80
$3(Q^{N_2}{}_A-Q^{O_2}{}_A)/Q_{O_2}$, Meyerhof oxidation quotient	5.8	5.3	4.0	2.5	2.3	2–6

[1] 0.013 M bicarbonate, 0.2 percent glucose. Metabolic Q values are in terms of "cubic millimeters"/milligram initial dry weight/hour, where 1 $mm.^3$ is 1/22,400,000 mol.

TABLE 2.—*Influence of paraphenylenediamine (PPDA), succinate, and cyanide on the oxygen consumption of tissue slices of transplanted mouse melanomas and other malignant tumors in Warburg-Okamoto-Ringer-glucose-phosphate medium* [1]

Tumor	Harding-Passey melanotic melanoma	Cloudman S91 melanotic melanoma	Algire S91A amelanotic melanoma	Earle L sarcoma	Barrett C3HBA adenocarcinoma	Malignant tumors in general (approximate range)
Q_{O_2}, oxygen consumption, r	2.0	5.0	5.0	5.5	12.0	2–12
Q_{O_2} in succinate (0.02 M)	4.0 [2] (100)	7.5 [2] (50)	7.5 [2] (50)	6.4 [2] (15)	.....	[2] (0–40)
Q_{O_2} in PPDA (0.02 M)	5.7 [2] (185)	18.0 [2] (260)	18.0 [2] (260)	9.0 [2] (65)	20.0 [2] (70)	[2] (0–120)
(0.05–0.1 M), R	16.0 [2] (700)	28.0 [2] (460)	31.0 [2] (520)	10.0 [2] (80)	26.0 [2] (115)	[2] (0–150)
Q_{O_2} in KCN (ca. 0.003 M) ±PPDA	<.5	<.5	<1	.....	<1	<1

[1] Usually 0.2 percent glucose and 0.013 M phosphate (pH 7.2), but little if any differences noted with zero glucose or 0.1 M phosphate where tested. PPDA (Eastman) and succinate and KCN added as aqueous solutions usually from side arm. Metabolic Q values are in terms of "cubic millimeters"/milligram initial dry weight/hour, where 1 $mm.^3$ is 1/22,400,000 mol.

[2] Values in parentheses indicate percentage increases of Q_{O_2} in treated compared with basal medium controls = $100\ (R-r)/r$.

In contrast with the foregoing similarity of results with the various tumors, the data of table 2 indicate that paraphenylenediamine causes a markedly greater stimulation of oxygen consumption in the case of the three melanomas than in the case of the Earle L sarcoma or Barrett C3HBA adenocarcinoma, and, it may be added, also six other mouse tumors reported upon by Roskelley, Mayer, Horwitt, and Salter (*11*) (sarcoma 180, Yale tumor No. 1, lymphosarcoma, two rhabdomyosarcomas, and a mouse "chest tumor" of Gardner), and malignant tumors generally (*5, 6, 9, 12, 13*). The same type of oxidative effect was obtained in table 2 with succinate as with paraphenylenediamine, but to a lesser degree. The increases with paraphenylenediamine are, in fact, those exhibited by essentially all normal adult and embryonic tissues, where a range of 100 to 2,000 percent increase in oxygen consumption may be noted with the methods employed, those reported on the basis of the methods of Rosenthal and Drabkin (*14*) being somewhat higher.

It is further noteworthy in table 2 that cyanide (ca. 0.003M) eliminated nearly all the respiration increase caused by paraphenylenediamine as well as almost all the basal respiration, in all the tumors studied.

DISCUSSION

It is probable that the cyanide inhibition of respiration above a Q_{O_2} value of no more than 0.5 or 1, in either the presence or absence of paraphenylenediamine, is due to inhibition of the cytochrome oxidase system, and, in particular, to combination by cyanide with cytochrome oxidase itself. This oxidase catalyzes the oxidation, by molecular oxygen, of paraphenylenediamine and endogenous substrate, as well as any glucose that may be involved. A good part of the small residual oxygen consumption that occurs in the presence of cyanide and paraphenylenediamine is probably due to endogenous substrate oxidized by oxidases other than cytochrome oxidase. It can barely be established with certainty, in fact, with the quantities of tissue usually employed, that paraphenylenediamine causes any notable increase in oxygen consumption in the presence of cyanide, since the small residual oxygen consumption in the presence of cyanide is nearly as great in the absence as in the presence of paraphenylenediamine. An appreciable action of cytochrome *b*, which is commonly regarded as autooxidizable and cyanide-insensitive, is not precluded in accounting for the residual, cyanide-insensitive oxygen consumption.

The marked stimulation of oxygen consumption caused by paraphenylenediamine in cyanide-free tissue slices of the melanomas, as compared with other tumors, may be interpreted as indicating that the ratio oxidized/reduced cytochrome *c* is considerably higher in these melanomas than in other tumors; for then, in the case of the melanomas, paraphenylenediamine would cause a greater diminution in this ratio and hence a greater observed percentage increase in oxygen consumption. This interpretation involves the usual assumptions (1) that paraphenylenediamine reduces cytochrome *c* but not cytochrome oxidase, (2) that cytochrome oxidase is in relative activity excess compared with cytochrome *c*, (3) that other cytochromes are not significantly involved, and (4) that the rate of oxygen consumption is approximately proportional to the concentration of reduced cytochrome *c*. Thus, if *r*, the normal rate of oxygen consumption in the absence of paraphenylenediamine, be taken as an approximate measure of the normal content of reduced cytochrome *c*; and *R* the rate of oxygen consumption in the presence of paraphenylenediamine as an approximate measure of the total cytochrome *c* content (measured in its completely reduced form); and $R-r$ as a measure of the normally occurring content of oxidized cytochrome *c*; then, the normal ratio of oxidized/reduced cytochrome *c*=(total cytochrome *c*−reduced cytochrome *c*)/reduced cytochrome *c*=$(R-r)/r$=the experimentally observed fractional stimulation of oxygen consumption by paraphenylenediamine. $100\ (R-r)/r$ is the percentage stimulation.

That the total cytochrome *c* content of the Harding-Passey tumor is somewhat less, and that of the Earle L sarcoma still less, than that of the other three tumors in table 2 is indicated by the corresponding order of relative rates of respiration, *R*, observed at high concentrations of paraphenylenediamine (16.0, 10.0, respectively, compared with 28.0, 31.0, and 26.0). This range of variation, however, is not extreme but represents an intermediate order of magnitude for tissues considered generally.

The contents of reduced cytochrome *c* in the various tumor slices under normal conditions of measurement (in the absence of paraphenylenediamine) would appear to vary in the decreasing order: Barrett C3HBA adenocarcinoma; Earle L sarcoma, S91A, and S91 melanomas; and Harding-Passey melanoma (*r* values, respectively, 12.0; 5.5, 5.0, 5.0; and 2.0). Similarly, the oxidized cytochrome *c* contents, under normal conditions of measurement would appear to vary in the decreasing order: S91A and S91 melanomas; Harding-Passey melanoma and Barrett C3HBA adenocarcinoma; Earle L sarcoma ($(R-r)$ values, respectively, 26.0, 23.0; 14.0, 14.0; and 4.5).

It would appear from homogenate studies (*15*) that the cytochrome oxidase activity of all three melanomas is approximately the same (1.1–1.5 Qo_2 units on a wet-weight basis), but high compared with similarly measured human tumors (0.2–1.0, average 0.4 units (*16*)) where considerable data (*11*) are available indicating a low oxidative response to paraphenylenediamine. It might be, then, that the relatively marked effect obtained with paraphenylenediamine in the three melanomas is partly an expression or result of this comparatively high cytochrome oxidase activity. This could be true if the usual assumption that cytochrome oxidase is in relative activity excess compared with cytochrome *c* were more fully realized in the melanomas than in the human tumors measured, and so permit more effective utilization of the increase in reduced cytochrome *c* content brought about by paraphenylenediamine; for, the higher the relative excess of cytochrome oxidase, the more readily would the maximum value of $(R-r)/r$ be realized for any given reduced cytochrome *c* level. Conversely, to the extent, especially in the human tumors, that cytochrome oxidase might not be in relative excess, *R* would attain less than its potential maximum (since the proportionality of rate with reduced cytochrome *c* content would fall off), and $(R-r)/r$ would be correspondingly low.

However, the cytochrome oxidase values of the mouse melanomas, compared with mouse tumors generally, are not high, but intermediate or even low, as reported for large series by Shack (*17*) and by Greenstein (*18*), and yet, as already indicated, where measurements have been made ((*11*) and table 2), other mouse tumors than the melanomas have all given low oxidative responses with paraphenylenediamine. It would seem, therefore, that the high oxidative responses obtained with the melanomas cannot be specifically correlated with cytochrome oxidase content. In this connection, there may be important cell structural aspects to be taken into consideration, affecting the turn-over numbers of both cytochrome *c* and cytochrome oxidase. Thus, the maximum cytochrome *c*-cytochrome oxidase activity in tumor homogenates was found (*16*) to be insufficient to account for the corresponding tissue slice respiration. It is possible to explain this as due either to destruction or loss of oxidase activity upon homogenation, decrease in cytochrome oxidase activity by loss of cell structure and integrity (decrease in turn-over number, or decrease in affinity between cytochrome oxidase and cytochrome *c*), or, much less probably, by major involvement of other oxidases than cytochrome oxidase. As is well known, the turn-over number of cytochrome *c* may be considerably larger intracellularly than extracellularly. Destruction of various oxidase activities upon homo-

genation appears to be a not infrequent phenomenon.

It remains to be determined how invariably the large stimulation of oxygen consumption caused by paraphenylenediamine in the case of the mouse melanoma tissue slices studied is characteristic of melanomas generally, particularly melanomas in man. Conceivably, also, other nonmelanoma tumors in mice or other species may be found, in spite of extensive contraindication so far, to yield large stimulations with paraphenylenediamine, especially if unusually high cytochrome oxidase activity should be found.

In any event, a high ratio of oxidized-reduced cytochrome *c* in tissue slices of the three melanomas, indicated by the marked stimulation of oxygen consumption by paraphenylenediamine, is further indicative of a higher level of oxidation-reduction potential within melanoma cells, either intracellularly throughout or at least locally in certain important cell areas. Whether or not this condition is even partly related to the ratio cytochrome *c*/cytochrome oxidase, it may well be connected, either as cause or result, with the presence of other oxidases known or suspected to be involved in melanin formation (*15, 16, 18, 19*), such as those acting upon dihydroxyphenylalanine (dopa) or tyrosine, substrates with which this paper has not been concerned experimentally.

The data in table 2 have established a differentiation between the three melanomas as a class compared with other tumors that have been measured similarly but have established no prominent differentiation between the three melanomas themselves. The biochemical reason or reasons for the evident lack of pigment formation in the amelanotic melanoma still remains to be ascertained. The reported relative lack of dopa oxidase or tyrosinase in homogenates (*15*) may suffice for explanation, or possibly a difference in mechanism of granule formation may be the determining factor. Conceivably a small but critical difference in internal oxidation-reduction potential may be involved, one that might be slightly too high in the amelanotic tumor, rather than possibly too low in nonmelanomas, for actual pigment formation.

It has been reported in homogenate studies (*15*) that the amelanotic melanoma, as compared with the melanotic melanomas, was nearly completely lacking in systems capable of oxidizing paraphenylenediamine. This would appear to be a result of the homogenation, for, as indicated in table 2, tissue slices of the amelanotic melanoma showed the highest paraphenylenediamine-oxidizing capacity of any of the tumors studied, melanotic or otherwise. A differential stability upon homogenation is indicated, if anything. In any event, with either type of melanoma, a great loss of paraphenylenediamine-oxidizing capacity occurs upon homogenation, amounting to 80 percent or more.

Similarly, the tissue-slice data of table 2 indicate no important difference in cyanide sensitivity of paraphenylenediamine stimulation as was reported for homogenates of the amelanotic as compared with the melanotic tumors (*15*). No cyanide-insensitive system in the melanotic tumors, capable of oxidizing paraphenylenediamine at an initial rate of the order of 0.4 $mm.^3$ per milligram wet weight per hour (or several cubic millimeters per milligram dry weight per hour for comparison with the data in table 2), was observed in the tissue slices. It would seem that such a system was emphasized by the homogenation, and that it might represent not any new enzyme but possibly a substrate, produced by cell destruction, that was susceptible to induced oxidation of cyanide-insensitive character.

It is not likely that changes in the biologic character of the tumors, occurring between the period of the earlier studies (*15*) and the present one, could account for the differences observed between the homogenate studies then and the tissue-slice studies now, for the existence of the cyanide-insensitive, paraphenylenediamine-oxidizing system in the two melanotic tumors has been confirmed quantitatively as to order in homogenates prepared during this investigation. Details of the mode of homogenation and measurement of result clearly affect the numerical values obtained, but it has been possible to obtain a homogenate from the amelanotic melanoma that was as active in order as those from the melanotic melanomas, in the presence of cyanide.

The foregoing differences indicated between tissue-slice and homogenate studies emphasize the need for further comparable studies in regard to substrates such as dihydroxyphenylalanine and tyrosine.

Various studies in the past have indicated little correlation between the relative rates of glycolysis and relative rates of growth of various comparable tumors. The present studies strongly suggest the desirability of making similar measurements with respect to relative magnitudes of oxidation responses to paraphenylenediamine, where an inverse relationship between growth rate and response might be found, either among similar or different tumors of the same or different species.

CONCLUSION

Tissue slices of the three transplantable mouse melanomas (Harding-Passey and Cloudman S91 pigmented melanomas and Algire S91A amelanotic melanoma) showed a metabolism that is

exhibited by malignant tumors generally in regard to aerobic and anaerobic glycolysis, oxygen consumption, respiratory quotient, and other derived quotients.

On the other hand, all three melanomas showed a much greater percentage stimulation of oxygen consumption by paraphenylenediamine than any other tumors tested to date, and the stimulation was in all cases essentially eliminated by cyanide. This finding of greatly enhanced stimulation of oxygen consumption by paraphenylenediamine offers the possibility of a biochemically new characterization and mode of diagnosis of melanomas, amelanotic as well as pigmented.

REFERENCES

(*1*) ALGIRE, G. H.: Growth and vascularization of transplanted mouse melanomas. Publ. New York Acad. Sc. (In press.)

(*2*) CRABTREE, H. G.: Observations on the carbohydrate metabolism of tumours. Biochem. J. 23: 536–545 (1929).

(*3*) BURK, D., SPRINCE, H., SPANGLER, J. M., KABAT, E. A., FURTH, J., and CLAUDE, A.: The metabolism of chicken tumors. J. Nat. Cancer Inst. 2: 201–240 (1941).

(*4*) BURK, D., SPRINCE, H., SPANGLER, J. M., BOON, M. C., and FURTH, J.: Metabolism of induced and spontaneous leukemias in mice. J. Nat. Cancer Inst. 3: 240–275 (1943).

(*5*) BURK, D., EARLE, W. R., and WINZLER, R. J.: Production of malignancy in vitro. VII. Metabolism and biotin vitamer content of tumors produced. J. Nat. Cancer Inst. 4: 363–372 (1944).

(*6*) KIDD, J. G., WINZLER, R. J., and BURK, D.: Comparative glycolytic and respiratory metabolism of homologous normal, benign, and malignant rabbit tissues, with particular reference to the benign virus papilloma (Shope) and a transplanted cancer derived therefrom (the V2 carcinoma). Cancer Research 4: 547–553 (1944).

(*7*) BURK, D.: On the biochemical significance of the Pasteur reaction and Meyerhof cycle in intermediate carbohydrate metabolism. Occas. Papers A. A. A. S. 4: 121–161 (1937).

(*8*) ———: A colloquial consideration of the Pasteur and neo-Pastuer effects. Cold Spring Harbor Symposia on Quant. Biol. 7: 420–459 (1939).

(*9*) ———: On the specificity of glycolysis in malignant liver tumors as compared with homologous adult or growing liver tissues. *In* A Symposium on Respiratory Enzymes, pp. 235–245. Univ. Wisconsin Press, Madison (1942).

(*10*) WARBURG, O.: Ueber den Stoffwechsel der Tumoren. J. Springer, Berlin (1926). Trans. by F. Dickens, The Metabolism of Tumours, Constable, London (1930); and R. N. Smith Co., New York (1931).

(*11*) ROSKELLEY, R. C., MAYER, N., HORWITT, B. N., and SALTER, W. T.: Studies in cancer. VII. Enzyme deficiencies in human and experimental cancer. J. Clin. Investigations 22: 743–751 (1943).

(*12*) CRAIG, F. N., BASSETT, A. M., and SALTER, W. T.: Artificial benignancy of neoplasm. VI. Observations on the oxidative behavior of tumors, artificially benign tumors, and homologous normal tissues. Cancer Research 1: 869–879 (1941).

(*13*) MAYER, N.: Studies in cancer. X. Oxidative capacity of tumors. Cancer Research 4: 345–348 (1944).

(*14*) ROSENTHAL, O., and DRABKIN, D. L.: The oxidative response of normal and neoplastic tissues to succinate and to *p*-phenylenediamine. Cancer Research 4: 487–494 (1944).

(*15*) GREENSTEIN, J. P., and ALGIRE, G. H.: Comparative oxidase activity of melanotic and amelanotic melanomas. J. Nat. Cancer Inst. 5: 35–38 (1944).

(*16*) GREENSTEIN, J. P., WERNE, J., ESCHENBRENNER, A. B., and LEUTHARDT, F. M.: Chemical studies on human cancer. I. Cytochrome oxidase, cytochrome *c*, and copper in normal and neoplastic tissues. J. Nat. Cancer Inst. 5:55–76 (1944).

(*17*) SHACK, J.: Cytochrome oxidase and *d*-amino acid oxidase in tumor tissue. J. Nat. Cancer Inst. 3: 389–396 (1943).

(*18*) GREENSTEIN, J. P.: Enzymes in normal and neoplastic animal tissues. *In* A. A. A. S. Research Conference on Cancer, pp. 192–222. Am. Assoc. Advancement Sc., Washington, D. C. (1945).

(*19*) HOGEBOOM, G. H., and ADAMS, M. H.: Mammalian tyrosinase and dopa oxidase. J. Biol. Chem. 145: 273–279 (1942).

(*20*) HERMANN, H., and BOSS, H. B.: Dopa oxidase activity in extracts from ciliary body and in isolated pigment granules. J. Cell. & Comp. Physiol. 26: 131–138 (1945).

FURTHER STUDIES ON THE RESPONSE OF STRAIN A FEMALE MICE TO SMALL AMOUNTS OF *o*-AMINOAZOTOLUENE[1]

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This report deals briefly with the results of two experiments designed to ascertain the minimum amount of *o*-aminoazotoluene capable of inducing hepatic lesions and pulmonary tumors in strain A female mice. In an earlier experiment (*1*), graduated subcutaneous doses of the crystalline compound moistened with glycerol were given to strain A females, and a single injection of approximately 10 mg. produced hepatic changes, hepatomas, and pulmonary tumors. Therefore, all injections reported here contained less than 10 mg.

MATERIALS AND METHODS

Because of difficulties attending the application of small amounts of crystalline material with any degree of accuracy, it was decided to dissolve the dye in olive oil. Previous work (*2*) had shown that repeated injections of olive-oil solutions of *o*-aminoazotoluene induced hepatic changes, hepatomas, and pulmonary tumors in strain A mice.

The *o*-aminoazotoluene was obtained from the Eastman Kodak Co. and used without further purification. The olive oil was of U. S. P. quality. Thirty-two milligrams of the compound was dissolved in each cubic centimeter of olive oil by heating gently, and the resultant solution was used to prepare lower doses by serial twofold dilutions so that each cubic centimeter of olive oil contained 32, 16, 8, or 4 mg. of the dye. A clean pipette was used for making each dilution.

EXPERIMENTAL PROCEDURE

All subcutaneous injections were made into the right axillary region by means of a 1-cc. syringe and a 20-gage needle, and each consisted of 0.25 cc. of olive oil containing 8, 4, 2, or 1 mg. of *o*-aminoazotoluene. The point of entrance of the needle through the skin was sealed with collodion to prevent escape of the oily solution. Oral administration was carried out by using a 1-cc. syringe and a blunt, curved, 20-gage needle, according to the technique described by Morris and Thompson (*3*).

Strain A virgin female mice from 2 to 3 months of age were used. They were housed on sawdust bedding in wooden cages, fed Purina laboratory chow, and given an unlimited supply of drinking water. Necropsies were performed upon all mice. The macroscopic appearance of the liver was recorded, a piece was prepared for histologic examination, and the presence and number of hepatomas and pulmonary tumors were noted.

In the first experiment, strain A animals procured from the Roscoe B. Jackson Memorial Laboratory received subcutaneous injections of 8, 4, 2, or 1 mg. of the dye, and hepatic reactions as well as hepatomas were found in those receiving the 4-mg. dose. Following this result, another experiment was started to compare the response of strain A females to subcutaneous or oral application of the compound. These were procured from Dr. J. J. Bittner, of the University of Minnesota. They were divided into two groups, one for subcutaneous injection and the other for oral administration.

Since the mice from both sources reacted similarly to graduated doses injected subcutaneously, the results of both experiments were combined and are summarized in tables 1 and 2.

TABLE 1.—*Induction of macroscopic hepatic reactions in strain A female mice following subcutaneous or oral administration of 1 to 8 mg. of o-aminoazotoluene dissolved in 0.25 cc. of olive oil*

[Numerator = Mice necropsied; Denominator = Mice with hepatic reactions]

Route of administration	Number of mice	Amount administered	Time after injection, in months: 3	4	6	7	9	12	14	15	Total	Incidence
		Mg.										*Percent*
Subcutaneous	31	8	2/0	4/1	8/8	2/2	4/4	7/7	....	4/4	31/26	84
Oral	15	8	2/1	....	4/3	....	4/4	5/5	....	....	15/13	87
Subcutaneous	31	4	....	2/0	8/3	2/2	4/3	8/7	1/1	6/5	31/21	68
Oral	16	4	....	....	5/2	....	4/3	5/5	....	2/2	16/12	75
Subcutaneous	29	2	....	2/0	4/0	....	4/0	8/0	1/0	10/2	29/2	7
Oral	15	2	....	....	2/0	....	4/1	4/2	1/1	4/3	15/7	47
Subcutaneous	28	1	....	....	3/0	1/0	2/0	5/0	4/0	13/0	28/0	0
Oral	16	1	....	....	....	....	3/0	4/0	3/0	6/0	16/0	0

TABLE 2.—*Induction of macroscopic pulmonary tumors and hepatomas in strain A female mice following subcutaneous or oral administration of 1 to 8 mg. of o-aminoazotoluene dissolved in 0.25 cc. of olive oil*

[Numerator = Mice with pulmonary tumors; Denominator = Mice with hepatomas]

Route of administration	Number of mice	Amount administered	Time after injection, in months[1]: 3	4	6	7	9	12	14	15	Total	Incidence
		Mg.										*Percent*
Subcutaneous	31	8	0/0	1/0	3/2	0/0	4/2	5/2	....	4/0	17/6	55/20
Oral	15	8	0/0	....	3/1	....	2/1	3/4	....	....	8/6	53/40
Subcutaneous	31	4	....	0/0	1/0	0/0	0/0	2/2	0/1	2/4	5/7	16/23
Oral	16	4	....	....	0/1	....	1/2	1/3	....	1/0	3/6	19/37
Subcutaneous	29	2	....	0/0	0/0	....	3/0	3/0	1/1	5/2	12/3	41/10
Oral	15	2	....	....	0/0	....	2/0	1/0	0/0	1/0	4/0	27/0
Subcutaneous	28	1	....	....	0/0	0/0	2/0	0/0	1/0	5/0	8/0	29/0
Oral	16	1	....	....	....	....	0/0	0/0	2/0	2/0	4/0	25/0

[1] See table 1 for number of mice necropsied each month.

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RESULTS

The findings presented in table 1 show that, under the conditions of these experiments, a single subcutaneous or oral injection of 8, 4, or 2 mg. of *o*-aminoazotoluene induced gross hepatic changes (*4*), but 1 mg. failed to do so. It is also seen that 7 of 15 mice given 2 mg. orally developed hepatic lesions whereas only 2 of 29 mice receiving the same amount subcutaneously did so. This suggests that oral administration was more effective than subcutaneous in producing hepatic lesions.

The data in table 2 are interpreted as showing that 8 mg. produced pulmonary tumors in both the injected and fed groups because these animals developed multiple tumors and had an average of five tumors per mouse. The incidence and number of pulmonary tumors in the lower dose groups indicated that amounts of less than 8 mg. failed to elicit this type of tumor. Most had but one pulmonary tumor, and this incidence could be expected in strain A mice.

Hepatomas occurred in some mice receiving 8 or 4 mg. Oral administration of 2 mg. failed to induce hepatomas, but three such tumors arose in mice receiving the same amount subcutaneously. These tumors were found in animals free of gross hepatic reactions. Two of these were examined histologically by Dr. A. J. Dalton, of the National Cancer Institute, who, on the basis of the characteristic appearance of the mitochondria and Golgi apparatus (*5*), concluded that they resembled hepatomas induced with *o*-aminoazotoluene. This suggests that the dye induced hepatomas in mice without producing gross hepatic changes.

DISCUSSION

The experiments confirmed previous findings that a single subcutaneous injection of small amounts of *o*-aminoazotoluene induced hepatic lesions and hepatomas in strain A female mice and, in addition, showed that a single oral administration of small amounts will give similar results. They also indicate that in strain A females the minimum amount capable of inducing hepatic lesions and hepatomas is between 1 and 2 mg. Smaller amounts may be effective if the mice are kept longer or belong to a more susceptible strain.

The investigations of Law (*6*) and Morosenskaya (*7*) who administered 10 mg. of the dye to mice were discussed in the previous paper (*1*). Both reported the induction of hepatomas, and in addition Law observed fibrosarcomas at the injection site while Morosenskaya found pulmonary tumors.

In the experiments reported here 8 mg. of the compound produced pulmonary tumors, but 4, 2, or 1 mg. failed to do so. Other tumors such as subcutaneous fibrosarcomas at the injection site and hemangio-endotheliomas, which are known to be induced by the compound (*2*, *4*), were not found. The liver of the strain A female mouse is apparently the organ most susceptible to tumor induction by small amounts of the dye when it is administered by the oral or subcutaneous routes.

The use of small amounts of carcinogens is desirable in some investigations. Small amounts may reveal the effects of factors such as strain, substrain, sex, age, nutrition, or solvent when large amounts may mask such influences. They may also be used advantageously in determining the additive effects, if any, of two carcinogens of the same or different nature, either physical or chemical.

Finally, in studies on the mechanism of tumor formation, large amounts of carcinogens may produce tissue and metabolic changes which are not involved in the production of malignancy. Small amounts of a carcinogen should reduce such changes to the minimum.

REFERENCES

(*1*) Andervont, H. B., and Edwards, J. E.: Response of strain A female mice to small amounts of *o*-aminoazotoluene. J. Nat. Cancer Inst. 3: 355–358 (1943).

(*2*) Andervont, H. B., and Edwards, J. E.: Carcinogenic action of two azo compounds in mice. J. Nat. Cancer Inst. 3: 349–354 (1943).

(*3*) Morris, H. P., and Thompson, J. W.: Convenient inexpensive device for quantitative hand feeding of mice. J. Nat. Cancer Inst. 1: 395–396 (1940).

(*4*) Andervont, H. B., Grady, H. G., and Edwards, J. E.: Induction of hepatic lesions, hepatomas, pulmonary tumors, and hemangio-endotheliomas in mice with *o*-aminoazotoluene. J. Nat. Cancer Inst. 3: 131–133 (1942).

(*5*) Dalton, A. J., and Edwards, J. E.: Mitochondria and Golgi apparatus of induced and spontaneous hepatomas in the mouse. J. Nat. Cancer Inst. 2: 565–575 (1942).

(*6*) Law, L. W.: The cancer producing properties of azo compounds in mice. Cancer Research 1: 394–401 (1941).

(*7*) Morosenskaya, L. S.: On the local effect of ortho-aminoazotoluene and the distant tumours elicited by it outside the liver. Arch. d. sc. biol. (U. S. S. R.) 56: 53–58 (1939).

ACCELERATION OF ENZYMATIC DESAMIDATION OF GLUTAMINE BY SEVERAL INORGANIC ANIONS[1]

By CHARLES E. CARTER, *assistant surgeon*, and JESSE P. GREENSTEIN, *chief biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The desamidation of glutamine in aqueous extracts of rat liver and kidney is accelerated by the addition of pyruvate (*1*). A similar acceleration of enzymatic desamidation of glutamine effected by inorganic phosphate, arsenate, and sulfate is reported in this paper.

MATERIALS AND METHODS

The glutamine used in this investigation was a gift from the Lederle Laboratories of the American Cyanamide Co. Isoglutamine (*2*) and chloroacetyl glutamine (*3*) were prepared by methods described.

Amide nitrogen was determined by hydrolysis of samples in 5 percent H_2SO_4 for 7 minutes or in 1N HCl for 1 minute at 100° C. Thus analyzed, the glutamine employed contained 95 percent of the theoretical value of amide nitrogen.

Ammonia determinations by aeration into acid followed by direct nesslerization were employed in most experiments to follow the enzymatic hydrolysis of the amide.

In several experiments the carbon dioxide evolved from the reaction of glutamine with chloramine-T was measured in Warburg vessels according to the technique described by Cohen (*4*).

EXPERIMENTS AND RESULTS

The enzymatic desamidation of glutamine in aqueous extracts of rat liver is accomplished at a slow rate. The addition of pyruvate to digests of glutamine and liver extract has been shown to result in an acceleration of desamidation (*1*), and as shown in table 1, a similar, rapid acceleration is produced by certain inorganic anions.

TABLE 1.—*Desamidation of glutamine, chloroacetyl glutamine, and isoglutamine in rat-liver extracts*

Salt	Glutamine	Chloroacetyl glutamine	Isoglutamine
	Micrograms NH_3-N	*Micrograms NH_3-N*	*Micrograms NH_3-N*
No added salt	19	8	146
Sodium phosphate	121	17	146
Sodium arsenate	88	15	146
Sodium sulfate	88	13	146
Sodium nitrate	19	8	146
Sodium bicarbonate	21	8	146

Rat liver was ground with sand, extracted with three volumes of water, and lightly centrifuged. All salts were present in final concentration of 0.06M. Digests consisted of 1 cc. of extract plus 1 cc. of salt in veronal buffer or veronal buffer alone plus 1 cc. of substrate (0.014M). The pH of the digests was carefully adjusted to 7.8. Incubation period was 1 hour at 37°. Results given in the table are expressed in micrograms NH_3 nitrogen. Under the foregoing conditions about 200 micrograms of amide nitrogen was available. All data were corrected for extract blanks.

In table 1 the specificity of the anion effect is illustrated by the failure of added inorganic salts to accelerate the desamidation of isoglutamine, whereas the peptide, chloroacetyl glutamine, behaves like glutamine, although its desamidation in the presence of the active anions is accomplished at a rate considerably less than that for glutamine under similar conditions.

Under the experimental conditions given in table 1 there was no hydrolysis of amide nitrogen to free ammonia in the absence of added enzyme or where a boiled liver extract was employed.

Asparagine is rapidly desamidated in liver extracts, but the rate of this reaction is not appreciably affected by the addition of inorganic salts which produce an acceleration of desamidation of glutamine. In kidney extracts where asparaginase activity is low, an anion effect on asparagine desamidation was not found. Kidney extracts rapidly desamidate glutamine, and as in liver extracts the rate of enzymatic desamidation is increased by certain anions.

Glutaminase activity is optimal at about pH 8.0 to 8.5, and the effect of added anions on the desamidation of glutamine in liver extracts was also found to be greatest in the range 8.0–9.0 (fig. 1). Under the conditions from which the data of figure 1 are derived, the pH optimum of glutaminase is not sharply defined because of the small magnitude of NH_3–N values.

Data are expressed in micrograms ammonia arising in 1 hour and at various pH values from hydrolysis of the amide group of glutamine. The digests consisted of 1 cc. of the liver extract containing the equivalent of 333 mg. tissue, plus 1 cc. of 0.014M glutamine. Salts were added in buffered solution to give the desired pH and final concentration of 0.06M. Incubation period was 1 hour at 37° C. All values were corrected for extract blanks.

That the inorganic anion effect is accomplished enzymatically is supported by the absence of the anion effect in extracts heated to 65° C. for 15 minutes and by the relation of the rate of desamidation to liver extract concentration at con-

[1] Received for publication February 24, 1947.

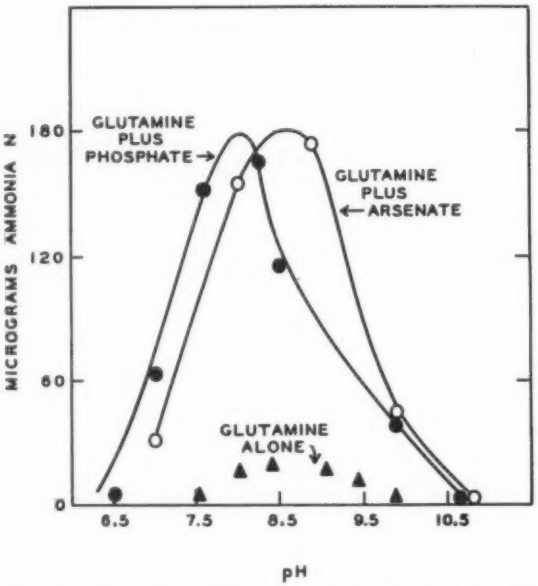


FIGURE 1.—Effect of pH on the desamidation of glutamine alone and in the presence of phosphate and of arsenate.

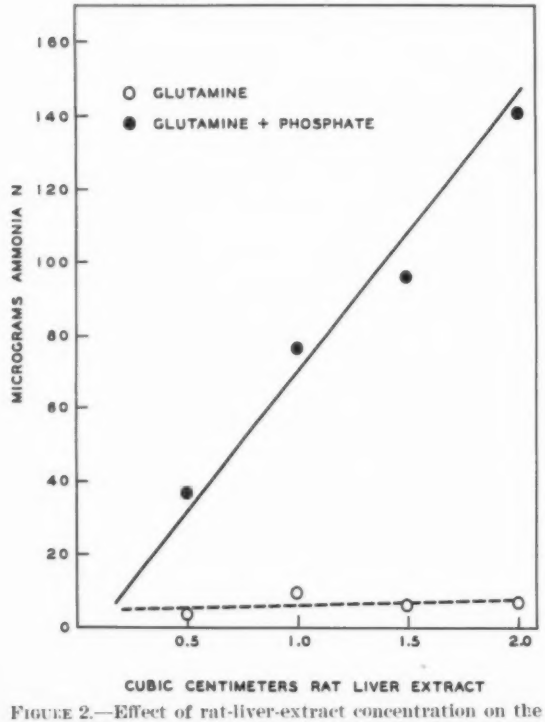


FIGURE 2.—Effect of rat-liver-extract concentration on the desamidation of glutamine alone and in the presence of phosphate.

stant phosphate concentrations (fig. 2). The data in figure 2 are expressed in terms of micrograms ammonia nitrogen resulting from the enzymatic hydrolysis of glutamine as a function of liver-extract concentration. The digests consisted of varying amounts of rat-liver extract at an initial concentration of 3 cc. of water per gram of tissue, made up to a constant volume of 2 cc. with distilled water, plus 1 cc. of 0.014M glutamine, and 1 cc. of phosphate buffer to give a final concentration of 0.06M and a pH of 7.2. At this pH the reaction is slower than at pH 8, thereby permitting the use of a wide range of extract concentration of high buffering capacity. The period of incubation was 1 hour at 37° C.

The relation of inorganic phosphate concentration to the rate of enzymatic desamidation of glutamine in dilute liver extracts is illustrated in figure 3. Digests consisted of 0.5 cc. of extract buffer solution prepared by extracting rat liver with three volumes of water and diluting with an equal volume of veronal buffer to give a final pH of 8.0, plus 0.5 cc. of varying concentrations of inorganic phosphate adjusted to pH 8 and 1 cc. of glutamine to give a final concentration of 6.8×10^{-3}M or 2.7×10^{-2}M. Data are corrected for extract blanks and are expressed in micrograms ammonia N arising from the enzymatic desamidation of glutamine. Period of incubation was 1 hour at 37° C.

The data of figure 3 show that the maximal rate of desamidation for two different concentrations of glutamine (6.8×10^{-3}M and 2.7×10^{-2}M) is approached at 0.03 M phosphate. The slope of the curves for the two different glutamine concentrations indicates that the lower glutamine concentration requires more phosphate to achieve a given fraction of optimal activity than does the higher glutamine concentration.

Because of a limited supply of glutamine the products of the anion-activated desamidations have not been isolated. In experiments employing inorganic phosphate this component has been found to remain constant throughout the course of the reaction. Hamilton (*5*) found that glutamine heated in aqueous solution at 100° C. for 90 minutes exhibited greater lability in the presence of phosphate than other inorganic ions. These conditions resulted in the formation of pyrrolidone carboxylic acid which by virtue of ring closure did not react with ninhydrin to evolve carbon dioxide. Chloramine-T, like ninhydrin, reacts with compounds having a free carboxyl group adjacent to a free α-amino group to evolve carbon dioxide, and this reaction has been employed by Cohen (*4*) in following transaminase activity. McIlwain (*6*) reported that pyrrolidone carboxylic acid did not evolve carbon dioxide when treated with chloramine–T.

In order to determine whether pyrrolidone carboxylic acid was a principal product of the phosphate-activated enzymatic desamidation, the chloramine-T reaction was carried out on the digest deproteinized by sulfuric acid-tungstic acid before and after incubation was employed. The pyrrolidone carboxylic acid used in these experiments was prepared by heating glutamine at 100° C. for 2 hours in the presence of 0.06M phosphate at pH 7.2.

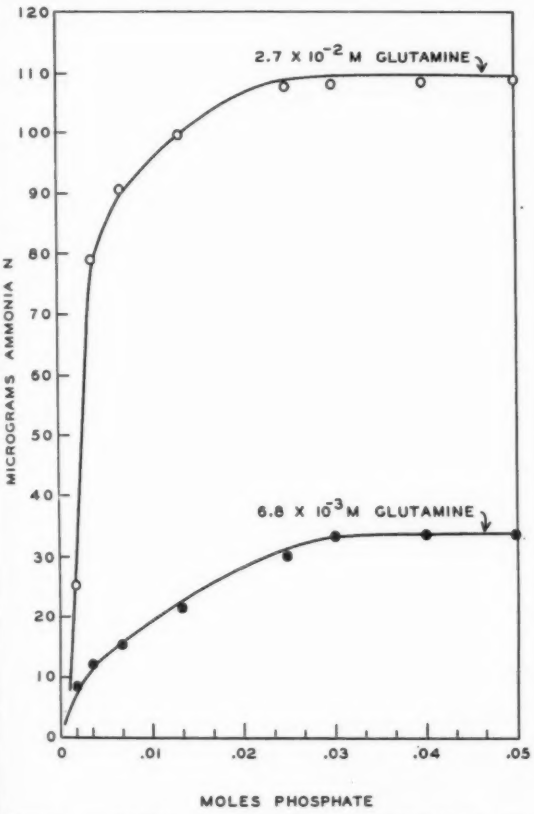


FIGURE 3.—Relation of phosphate concentration to enzymatic desamidation of glutamine.

Digests consisted of 1 cc. of rat-liver extract equivalent to 166 mg. tissue, 1 cc. of 0.014M glutamine or pyrrolidone carboxylic acid and 1 cc. of 0.06M phosphate. The final pH of the mixture was 7.8. Blanks contained water in place of glutamine. The incubation period was 2 hours at 37° C. All data were corrected for blanks. The determination of CO_2 evolved from the chloramine-T reaction was carried out in Warburg vessels according to Cohen (*4*).

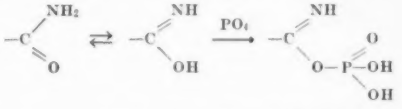
The carbon dioxide evolved in the digests containing pyrrolidone carboxylic acid varied initially in several experiments from 40 to 100 microliters, and after 2 hours of incubation from 52.5 to 100 microliters. The carbon dioxide evolved in the digests containing glutamine varied in several experiments initially from 451 to 650 microliters, and after 2 hours of incubation from 680 to 787 microliters. Determination of the amount of ammonia N and residual amide nitrogen indicated about 85 percent hydrolysis. The theoretical value for glutamine on the basis of Cohen's value for glutamic acid (*4*) is 627 microliters carbon dioxide.

These data show that the product of the phosphate-activated desamidation of glutamine reacts with chloramine-T to evolve CO_2 in amounts slightly higher than glutamine, thereby eliminating pyrrolidone carboxylic acid as a product of the reaction. Furthermore, pyrrolidone carboxylic acid when incubated with liver does not yield a product which reacts with chloramine-T to evolve CO_2, a finding which indicates that pyrrolidone carboxylic acid is not an intermediate product. Presumably glutamic acid and ammonia are the principal products of the anion-activated desamidation of glutamine by aqueous liver extracts.

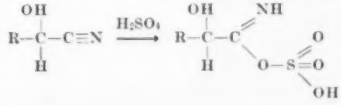
The anion acceleration of enzymatic desamidation of glutamine in aqueous liver extracts is diminished but not destroyed by dialysis, providing the pH of the dialyzate is maintained at 7.5 to 8.0.

DISCUSSION

The acceleration of desamidation of glutamine in aqueous rat-liver extracts by phosphate, arsenate, and sulfate may involve several mechanisms. Of these the most probable are (1) the formation of a labile intermediate combination of glutamine and anion, and (2) an anion activation of glutaminase. The possibility of tautomerism of the amide group of glutamine to an imino group as illustrated suggests that an esterified imino compound could arise as follows:



Karrer (*7, p. 178*) cites the following reaction where an imide-sulfuric acid ester has been isolated from the acid hydrolysis of α-hydroxy nitriles.



On the other hand, the fact that the optimum effect of phosphate and arsenate occurs at nearly

the same pH optimum as that of glutaminase, suggests that the effect of these ions may be due to an augmentation of the activity of this enzyme. The possibility that both mechanisms might occur is not excluded.

SUMMARY

Aqueous extracts of rat liver which possess little glutaminase activity are capable of rapidly desamidating glutamine in the presence of added phosphate, arsenate, or sulfate in the usual order of their effectiveness. Liver extracts dialyzed against distilled water lose the capacity to desamidate glutamine in the presence of the active anions. If the pH of the dialyzate is maintained at 7.5–8.0 by bicarbonate most of the activity is retained.

The pH at which the inorganic anion effect is optimal in liver extracts is 8.0, the optimum of glutaminase activity. Pyrrolidone carboxylic acid has been excluded as a product of the reaction by use of the chloramine-T method.

REFERENCES

(*1*) Greenstein, J. P., and Carter, C. E.: Influence of α-keto acids on desamidation of amino acid amides. J. Nat. Cancer Inst. 7: 55–60 (1947).

(*2*) Bergmann, M., and Zervas, L.: Über ein allegemeines Verfahren der Peptide-Synthese. Ber. d. deutsch. chem. Gesellsch. 65: 1192–1201 (1932).

(*3*) Price, V. E., and Greenstein, J. P.: Studies on effect of pyruvate on desamidation of glutamine, asparagine, and related compounds. J. Nat. Cancer Inst. 7: 275–279 (1947).

(*4*) Cohen, P. P.: Transamination with purified enzyme preparations (transaminase). J. Biol. Chem. 136: 565–584 (1940).

(*5*) Hamilton, P. B.: Gasometric determination of glutamine amino acid carboxyl nitrogen in plasma and tissue filtrates by ninhydrin-carbon dioxide method. J. Biol. Chem. 158: 375–395 (1945).

(*6*) McIlwain, H.: Metabolism and functioning of vitamin-like compounds. 3. Products of decomposition of glutamine during streptococcal glycolysis. Biochem. J. 40: 460–464 (1946).

(*7*) Karrer, P.: Organic chemistry. Elsivier Publishing Co., New York (1946).

EFFECTS OF ACIDITY AND OF HEATING ON THE CAPACITY OF RAT LIVER EXTRACTS TO DESAMIDATE GLUTAMINE AND ASPARAGINE[1]

By MAURICE ERRERA,[2] *research fellow*, and JESSE P. GREENSTEIN, *chief biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Glutamine is desamidated relatively slowly by aqueous extracts of rat liver. When pyruvate (*1–4*) or phosphate (*5*) is added to the digest, the rate of desamidation is considerably increased. The pH-activity curve of glutaminase reaches a maximum at pH 8.0. The effect of phosphate on the rate of desamidation of glutamine also reaches a maximum at pH 8.0 (*5*). At pH 6.0 there is no evidence of desamidation of glutamine alone or in the presence of added phosphate (*5*), but in the presence of added pyruvate there is a considerable desamidation of glutamine (*2*). The pH-activity curve for the effect of pyruvate on the desamidation of glutamine reaches a peak at about pH 7.2 (*2*).

Addition of pyruvate to digests of rat-liver extract with asparagine results in an increased rate of desamidation (*1–4*), but addition of phosphate has no effect on the desamidation of this amide (*5*). Pyruvate thus has the same effect on glutamine and on asparagine, but phosphate appears to affect only the desamidation of glutamine.

In the hope of further separating and distinguishing the effects of pyruvate and phosphate on the desamidation of acid amides, we subjected the rat-liver extracts used as the source of the enzymes to various procedures involving acidifying at various pH values and heating for various time intervals prior to digestion with the substrates at pH 8.0 and at 37° C.

MATERIALS AND METHODS

The preparation of aqueous rat-liver extracts, of substrates, and the methods of conducting the enzymatic analysis have been described (*1–5*). The heating was done by immersing freshly prepared extracts in a constant-temperature bath for various periods. The extract was then immediately chilled, warmed to room temperature, and aliquots were employed for the enzymatic digests. The acidifying procedures were performed by adding acetic acid or dilute hydrochloric acid slowly, while stirring, to the chilled extract. The pH of the extracts prior to heating or to the addition of acid was 6.7–6.9. After treating with heat or acid and letting stand for designated periods, the extracts were brought to pH 8.0 by addition of veronal acetate-sodium hydroxide buffer. The digests consisted of 1 cc. of treated or untreated extract at a concentration equivalent to 333 mg. tissue, 1 cc. veronal buffer, 1 cc. 0.014M glutamine or asparagine, and 1 cc. of either 0.025M sodium pyruvate or 0.022M phosphate. Blanks contained water in place of either the amides, the pyruvate or phosphate, or both, and the readings are corrected for these blanks. The digestion period was 4 hours at 37° C. The final pH at the end of the reaction was 7.7–7.8.

RESULTS

Acid Stability of Rat-Liver Extracts

Figure 1 illustrates the pH-activity curves of glutamine, of glutamine plus added phosphate, and of glutamine plus added pyruvate, all with rat-liver extracts. The data for glutamine and for glutamine plus pyruvate are from Gonçalves and Greenstein (*2*), those for glutamine plus phosphate from Carter and Greenstein (*5*). The curves have been placed on one figure for ready reference. It will be noted that the optimum phosphate effect on desamidation of glutamine is nearly the same as that of glutaminase, whereas the optimum pyruvate effect is definitely more

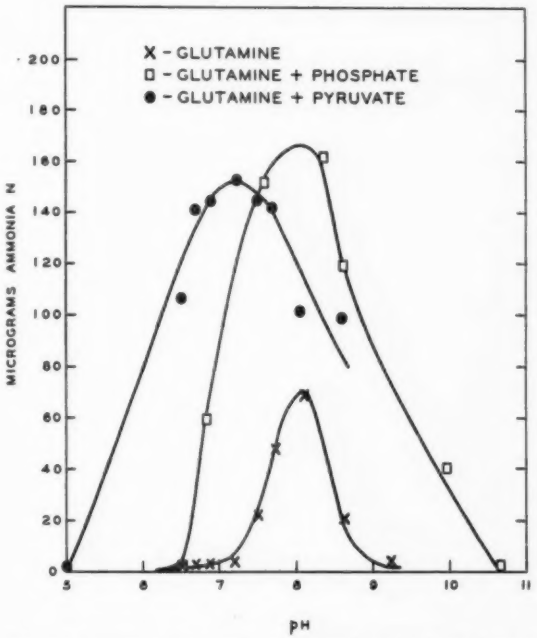


FIGURE 1.—Amide nitrogen as ammonia split from digests of rat-liver extracts with glutamine, glutamine plus pyruvate, and glutamine plus phosphate, as a function of the pH of the digests.

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[2] On leave from University of Brussels, Belgium.

acid. At pH 6.0 there is no desamidation of glutamine, alone or in the presence of added phosphate, but there still is a considerable desamidation of this amide in the presence of added pyruvate. At pH 8.0 the rate of desamidation of glutamine is much faster in the presence of phosphate than of pyruvate (*5*). In this connection, it should be remembered that the curves in figure 1 for glutamine and for glutamine plus pyruvate show results obtained after 4 hours of incubation at 37° C., whereas the curve for glutamine plus phosphate shows results obtained after 1 hour of incubation at this temperature.

Figure 2 gives data relating to the activity of rat-liver extracts at various pH values (veronal buffers) under the same conditions as those obtained for glutamine, with and without pyruvate (*2*) (cf. fig. 1). The higher activity of asparaginase in rat-liver extract compared with that of glutaminase is revealed. The effect of pyruvate on the desamidation of asparagine is best revealed at about pH 6.7 (*1*). At pH 8.0, the optimum for asparaginase activity, the asparagine is itself desamidated to about 75 percent, hence the effect of pyruvate is not so obvious at this pH.

Figure 3 depicts the stability at various pH values of fresh aqueous extracts of rat liver (3 cc. of water per gram of tissue). The extracts were brought to the pH values designated, kept for 2 hours at 25° C., and 1 cc. aliquots brought to pH 8.0 by the addition of 1 cc. of veronal acetate-dilute sodium hydroxide buffer. To this were added 1 cc. of 0.014M glutamine and 1 cc. of either 0.025M pyruvate or 0.022M phosphate. The digests were then incubated for 4 hours at 37°. The final pH was 7.7–7.8 in all tubes. It is noted from figure 3 that destruction of the capacity of the extract to desamidate glutamine, alone or in the presence of phosphate, increases with decreasing pH far more than its capacity to desamidate glutamine in the presence of pyruvate. After 2 hours of standing at pH 5, the capacity of the rat-liver extract to desamidate glutamine with or without added phosphate at pH8 is completely lost, whereas the capacity to desamidate glutamine in the presence of pyruvate is decreased by only 20 percent.

These phenomena are further emphasized in figure 4. The data were obtained with fresh rat-liver extracts allowed to stand at 25° C. at pH 5.5 for various lengths of time before being brought to pH 8.0 with veronal buffer and treated with substrate solutions as described for data given

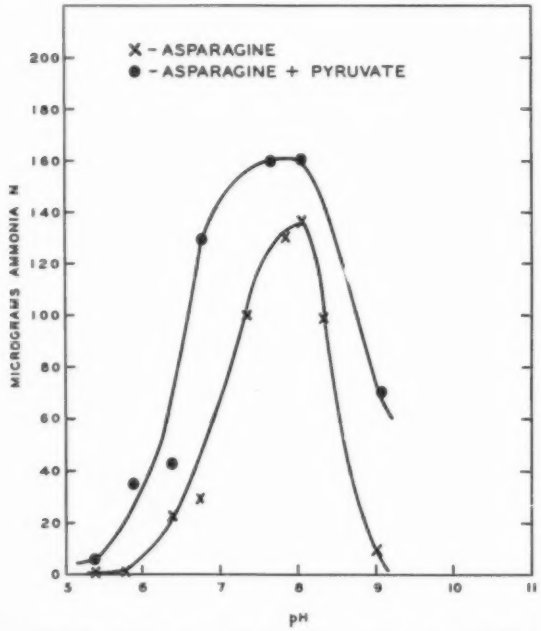

FIGURE 2.—Amide nitrogen as ammonia split from digests of rat-liver extracts with asparagine, and asparagine plus pyruvate, as a function of the pH of the digests.

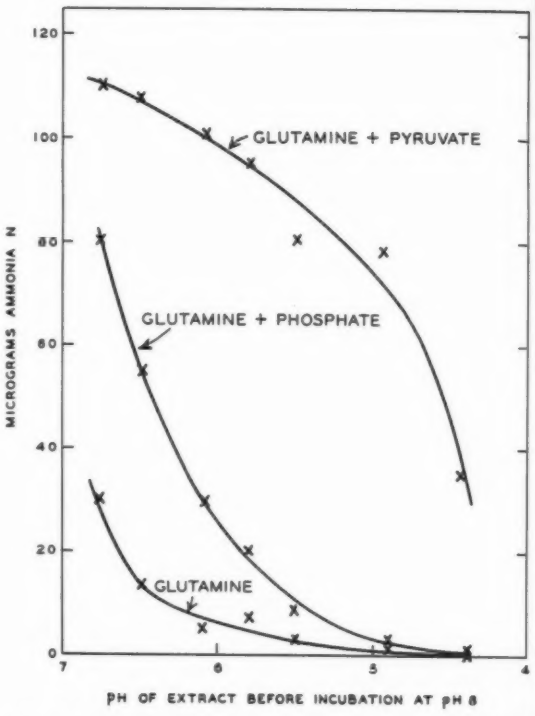

FIGURE 3.—Amide nitrogen as ammonia split at pH 8.0 from digests of glutamine, glutamine plus pyruvate, and glutamine plus phosphate with aqueous rat-liver extracts which had stood for 2 hours at various pH values prior to digestion of the substrates. All extract aliquots taken from the same stock extract solution.

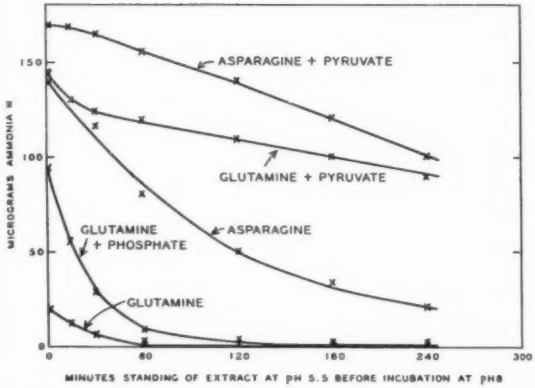

FIGURE 4.—Amide nitrogen as ammonia split at pH 8.0 from digests of glutamine, glutamine plus pyruvate, glutamine plus phosphate, asparagine, and asparagine plus pyruvate with aqueous rat-liver extracts which had stood at pH 5.5 for varying lengths of time prior to digestion of the substrate. All extract aliquots taken from the same stock extract solution.

in figure 3. Except for periods during which the extract stood prior to the final digestion with the substrates, the conditions of the experiments described in figure 4 were the same as those described in figure 3. The data in figure 4 reveal that even after 4 hours at pH 5.5, the capacity of the extract at pH 8.0 to desamidate glutamine or asparagine in the presence of pyruvate had decreased relatively little; yet after only about 1 hour the capacity at pH 8.0 to desamidate glutamine alone or in the presence of phosphate had virtually disappeared, while its capacity to desamidate asparagine alone had markedly decreased.

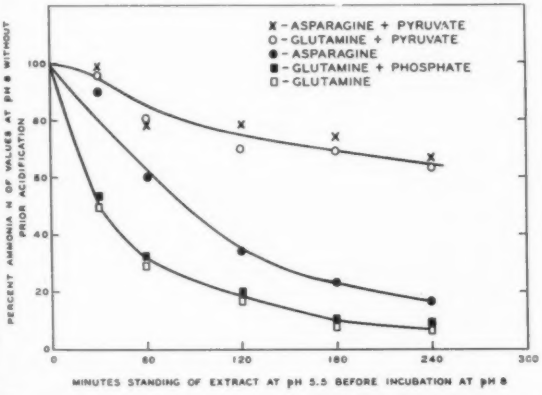
X - ASPARAGINE + PYRUVATE
O - GLUTAMINE + PYRUVATE
● - ASPARAGINE
■ - GLUTAMINE + PHOSPHATE
□ - GLUTAMINE
PERCENT AMMONIA N OF VALUES AT pH 8 WITHOUT PRIOR ACIDIFICATION
MINUTES STANDING OF EXTRACT AT pH 5.5 BEFORE INCUBATION AT pH 8

FIGURE 5.—Percent of activity at pH 8.0 of the capacity of rat-liver extracts pre-acidified for 2 hours at pH 5.5 and then incubated at pH 8.0 to desamidate glutamine or asparagine, glutamine plus pyruvate or phosphate, and asparagine plus pyruvate. Data based on figure 4.

The data on acid stability of rat-liver extracts are collected in figure 5 as percent of the respective values of unacidified extract at pH 8 given by various acidified extracts which had stood at pH 5.5 for different intervals before being brought to pH 8.0. The stability of enzymes in the extracts concerned with the desamidation of glutamine or asparagine in the presence of pyruvate is nearly the same, and with the desamidation of glutamine alone or in the presence of phosphate is also nearly the same.

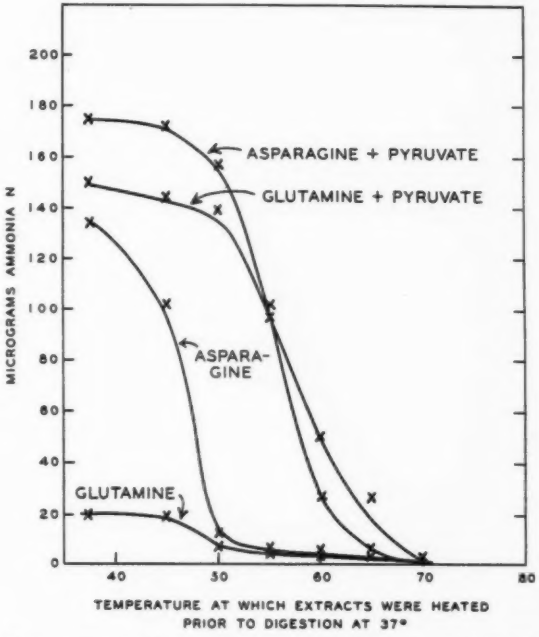

FIGURE 6.—Amide nitrogen as ammonia split at pH 8.0 from digests of amino acid amides alone and in the presence of pyruvate with aqueous rat-liver extracts heated for 10 minutes at various temperatures prior to digestion of the substrates. All extract aliquots taken from the same stock extract solution.

HEAT STABILITY OF RAT-LIVER EXTRACTS

Figure 6 gives data on the effect of heating aliquots of the same stock extract solution (3 cc. of water per gram of tissue) for 10 minutes at various temperatures. Following this the extracts were chilled, warmed to room temperature, mixed with veronal buffer to bring to pH 8.0, substrate solution was added, and the mixtures were incubated for 4 hours at 37° C. The total volume of the digests, 4 cc., and their composition were the same as in experiments depicted in figure 3. The final pH was 7.7–7.8.

Figure 6 shows that when preheated at 50° C., the capacity of the extracts to desamidate the

amides alone is practically lost, whereas the capacity to desamidate the amides in the presence of pyruvate is little affected. Above this temperature there is a rapid decrease in the latter capacity, but even when preheated at 60° C. there is an appreciable residual capacity. At 70° C. this capacity is completely gone. These phenomena are better revealed by asparagine than by glutamine, since asparaginase activity of liver is much higher than glutaminase activity. To illustrate the point still further, figure 7 gives the percentage of original

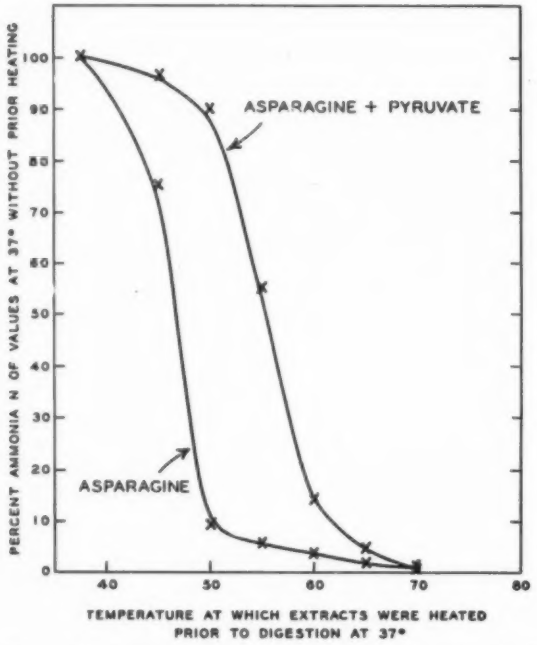

FIGURE 7.—Percent of activity at 37° C. of the capacity of rat-liver extracts preheated for 10 minutes at various temperatures to desamidate asparagine alone and in the presence of pyruvate at 37° C. Data based on figure 6.

asparagine-desamidation activity at 37° C. as a function of the heat stability of the extract prior to digestion. The data in figure 7 were derived from those shown in figure 6. The ordinate in figure 7 is given as the ratio multiplied by 100 of amide ammonia N found in digests containing preheated extract at various temperatures to that of digests preheated for the same period of time (10 minutes) at 37° C. In brief, each point on the curves in figure 7 represents the percent of the first point on the corresponding curves in figure 6. The capacity of the extract preheated at 50° C. to desamidate asparagine has dropped to about 10 percent of the original; the capacity of the same extract to desamidate asparagine in the presence of pyruvate has dropped only to 90 percent of its original value.

Figure 8 reveals the capacity of fresh aqueous extracts of rat liver (3 cc. of water per gram of tissue) preheated for varying lengths of time at 55° C. to desamidate glutamine at 37° C. alone and in the presence of either added phosphate or added pyruvate. Except for the length of preheating, experimental conditions were the same as those described. The digests consisted of 1 cc. of preheated extract chilled after heating and then brought to room temperature, 1 cc. of veronal buffer to bring the mixture to pH 8.0, 1 cc. of 0.014 glutamine, and 1 cc. of either 0.022M phosphate or 0.025M pyruvate. The digestion period was 4 hours at 37° C. Although preheating the extract at 55° C. for only 5 minutes before cooling, mixing with the substrates, and digesting for 4 hours at 37° C. nearly destroys the capacity of the extract to desamidate glutamine alone and in the presence of added phosphate, the capacity of the same extract to desamidate glutamine in the presence of added pyruvate is appreciable even after it has been preheated for a half hour.

DISCUSSION

From the data given in this and preceding papers (*1–4*), it appears probable that the effect of pyruvate on the desamidation of glutamine and of asparagine is not concerned with glutaminase or asparaginase activity, respectively. The pH

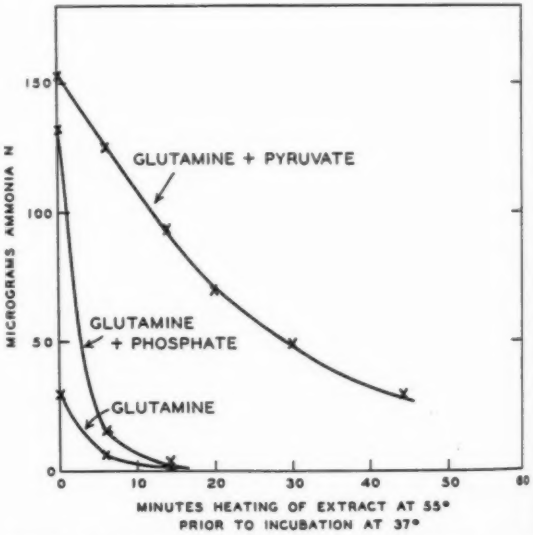

FIGURE 8.—Amide nitrogen as ammonia split at pH 8.0 from digests of glutamine with rat-liver extracts preheated for varying lengths of time at 55° C. before digesting at 37° C. with substrates. All extract aliquots were taken from the same stock extract solution.

optimum of the activity of glutaminase is more alkaline than that of the effect of pyruvate on the desamidation of glutamine. The stability of tissue extract in acid is much greater in the case of the pyruvate effect than in that of glutaminase or asparaginase. At temperatures which completely inactivate glutaminase and asparaginase activity in liver extracts, the capacity of such extracts to desamidate glutamine and asparagine in the presence of pyruvate is almost completely retained. The data in figure 1 were obtained on digests of glutamine in the presence and absence of added pyruvate. The relatively high activity of the pyruvate effect at pH 6.0, for example, where glutaminase activity is nil, might be due to some protective effect of added pyruvate on glutaminase in acidified extract. This explanation may, however, be ruled out by the data given in figures 3 and 4 obtained on extracts pretreated with acid before alkalinizing and mixing with the substrates. It may suggest instead that the different acid stability revealed when glutamine or glutamine plus pyruvate was added is due to involvement of different enzymatic mechanisms in each case. The different heat stability of the extract for asparagine and for asparagine plus pyruvate further emphasizes this point. That there is no appreciable destruction of asparaginase or of the pyruvate effect on asparaginase desamidation in liver extracts at pH 6.8 is shown by the linear and progressive character of the curves for these activities over a 4-hour period at 37° C. (*3*).

It seems highly probable, therefore, that desamidation of glutamine and of asparagine in the presence of pyruvate is effected by the formation of some labile intermediate compound between the amino acid amide and pyruvate. The nature of this hypothetical intermediate is not known at the present time. In the first paper in this series, Greenstein and Carter (*1*) suggested that the most probable type of intermediate between amide and keto acid would be of the nature of a dehydropeptide, giving reasons for this belief. In our opinion this explanation is still the most likely at the present time to account for the invariably greatly increased rate of desamidation of glutamine and of asparagine in the presence of added pyruvate (*1–4*). According to this explanation, two enzymatic steps are involved in this pyruvate effect, namely, (1) a condensation between amide and carbonyl group of the keto acid to form a dehydropeptide, followed by (2) splitting of the dehydropeptide to yield the corresponding amino acid, ammonia, and pyruvate. The keto acid is not consumed by the reaction but is completely regenerated (*1–2*), and the ammonia which appears is accounted for by a corresponding decrease in the amide nitrogen of the amide. To prove the validity of the foregoing explanation, the intermediate dehydropeptide must be either isolated or recognized during the course of the reaction. Attempts to note a change in the ultraviolet light absorption at 2500 Å in digests of glutamine with pyruvate in rat-liver extracts have been negative. Although the high characteristic absorption of dehydropeptides in this spectral range is not observed in such digests (*6*), this can be explained by the powerful dehydropeptidase activity in liver extracts (*6–7*) which probably results in the splitting of the dehydropeptide as fast as it is formed.

The effect of pyruvate on desamidation of amino acid amides probably is also different from the effect of phosphate. Both pyruvate and phosphate addition to liver extract results in an increased rate of desamidation of glutamine (*1–5*), but whereas pyruvate also increases the rate of desamidation of asparagine, phosphate has no effect on the desamidation of this amino acid amide (*5*). The pH-activity range, acid stability, and heat stability of rat-liver extracts in respect to the desamidation of glutamine tend to be similar in the presence and in the absence of added phosphate. Considered together with the high acceleration of glutamine desamidation effected by this anion, a rate far greater than that of pyruvate at equivalent concentration, the effect of added phosphate in liver extracts appears to be mainly a direct activation of glutaminase activity. In the absence of established evidence, however, the possibility of some labile, intermediate phospho-imino ester, formed by union between phosphate and amide group, as suggested by Carter and Greenstein (*5*), may also be invoked to explain the accelerating effect of phosphate on the desamidation of glutamine.

SUMMARY

Aqueous extracts of rat liver treated with acid to yield varying pH valves and allowed to stand for varying lengths of time before bringing to pH 8.0, mixing with glutamine or asparagine in the presence or absence of added pyruvate, and incubating at 37° C. for 4 hours, revealed as the pH decreased, a rapid and progressive decrease in their capacity to desamidate glutamine or asparagine alone. Their capacity to desamidate glutamine or asparagine in the presence of added pyruvate was preserved to an appreciable degree.

Aqueous extracts of rat liver when heated at various temperatures for different periods of time before chilling, warmed to room temperature, and incubated with glutamine or asparagine in the presence or absence of added pyruvate or phosphate, revealed, as temperature increased, a rapid and progressive decrease in their subsequent capacity to desamidate glutamine alone or in the presence of added phosphate, and to desamidate

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asparagine alone. Their capacity to desamidate either glutamine or asparagine in the presence of added pyruvate was preserved to an appreciable degree.

It is probable that pyruvate accelerates the desamidation of glutamine and asparagine in rat-liver extracts by a mechanism independent of glutaminase or asparaginase activity. It also appears probable that phosphate accelerates the desamidation of glutamine by a direct activation of glutaminase, but other mechanisms are not excluded.

REFERENCES

(*1*) GREENSTEIN, J. P., and CARTER, C. E.: Influence of α keto acids in the desamidation of amino acid amides. J. Nat. Cancer Inst. 7: 57–60 (1946).

(*2*) GONÇALVES, J. M., and GREENSTEIN, J. P.: Enzymatic desamidation of glutamine in the presence of pyruvate furnished by concomitant reactions. J. Nat. Cancer Inst. 7: 269–274 (1947.)

(*3*) PRICE, V. E., and GREENSTEIN, J. P.: Studies on the effect of pyruvate on the desamidation of glutamine, asparagine, and related compounds. J. Nat. Cancer Inst. 7: 275–279 (1947).

(*4*) GONÇALVES, J. M., PRICE, V. E., and GREENSTEIN, J. P.: Desamidation of amino acid amides in rat-liver extracts of varying concentration. J. Nat. Cancer Inst. 7: 281–284 (1947).

(*5*) CARTER, C. E., and GREENSTEIN, J. P.: Acceleration of enzymatic desamidation of glutamine by several inorganic anions. J. Nat. Cancer Inst. 7: 433–436 (1947).

(*6*) CARTER, C. E., and GREENSTEIN, J. P.: Spectrophotometric determination of dehydropeptidase activity in normal and neoplastic tissues. J. Nat. Cancer Inst. 7: 51–56 (1946).

(*7*) GREENSTEIN, J. P., and LEUTHARDT, F. M.: Degradation of cystine peptides by tissues. IV. Dehydropeptidase activity in normal and neoplastic tissues. J. Nat. Cancer Inst. 6: 197–201 (1946).

ENZYMATIC HYDROLYSIS OF ACETYLDEHYDROALANINE [1]

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INTRODUCTION

Peptides of α-aminoacrylic acid (dehydroalanine) with glycine or chloroacetic acid as the acyl radical are hydrolyzed in aqueous extracts of kidney and liver to yield products which include pyruvic acid and ammonia (*1–5*). The enzymatic system responsible for the hydrolysis of this class of compounds was designated by Bergmann and Schleich as dehydropeptidase (*6, 7*).

Glycyldehydroalanine is hydrolyzed by all the tissues studied, including normal and neoplastic, and in all species investigated (*1–5*). Chloroacetyldehydroalanine, on the other hand, is hydrolyzed with appreciable velocity, only in extracts of kidney, liver, and pancreas of all the tissues studied. On the basis of this distribution, it was postulated that at least two distinct dehydropeptidases existed. One designated as dehydropeptidase I existed in high activity in all animal tissues, and for it the substrate glycyldehydroalanine could be employed (*4*). The other, designated as dehydropeptidase II, possessed a much more limited distribution, and for it the substrate chloroacetyldehydroalanine could be employed.

The present paper is concerned with the susceptibility to enzymatic attack of still a third type of dehydropeptide, acetyldehydroalanine, in which the acyl radical possesses no polar substituent. Comparison of the rate of splitting of this substrate in rat-kidney extracts with that of the corresponding glycyl and chloroacetyl peptides is described.

MATERIALS AND METHODS AND EXPERIMENTAL PROCEDURE

Aqueous extracts of rat tissues were prepared as described (*4, 5*). Each cubic centimeter contained the equivalent of 333 mg. of fresh tissue. The digests, unless noted otherwise, consisted of 1 cc. of extract plus 2 cc. of borate buffer at pH 8.2, and 1 cc. of either water or 0.025M peptide. The peptide solution was neutralized with dilute sodium hydroxide before use. The initial pH of the digest was 8.0, and the final pH was about 7.8–8.0.

Acetyldehydroalanine was prepared by heating diacetaminopropionic acid in glacial acetic acid solution according to the method of Bergmann and Grafe (*8*). The product was crystallized from absolute methanol from which solvent it separates on chilling in flat, glistening plates. The diacetaminopropionic acid was prepared by the method described (*9*); the yield of this product is about 80 percent if purified acetonitrile and pyruvic acid are employed.

The hydrolysis of the substrates was followed by measuring the ammonia produced at the end of the incubation period and by the amount of pyruvate produced (*1–4*). Recovery experiments showed that 90–95 percent of added ammonia or pyruvic acid could be recovered from dialyzed extracts of rat liver and kidney after 4 hours of incubation. Acetyldehydroalanine was also characterized by its absorption in the ultraviolet region of the spectrum, and its hydrolysis in aqueous kidney extracts was followed spectrophotometrically by the method previously described (*5*). The substrate was neutralized before use with dilute sodium hydroxide. It is stable in aqueous solution for several weeks.

The glycyldehydroalanine and chloroacetyldehydroalanine studied were part of the material reported earlier (*1–4*), and acetyldehydrophenylalanine was furnished by Dr. J. White.

A few experiments were similarly performed with acetyldehydrophenylalanine as substrate.

RESULTS

Table 1 gives the data on the ammonia and pyruvate produced from acetyldehydroalanine in fresh aqueous extracts of rat tissues. The data are corrected for the extract blanks. Period of incubation was 4 hours at 37° C.

Kidney is by far the most active tissue in hydrolyzing acetyldehydroalanine, with liver next in order, while spleen, pancreas, brain, and muscle reveal little or no activity. The molar ratio of ammonia to pyruvic acid which appears in the digests at the end of the incubation period is close to unity, a finding consistent with that noted previously for glycyldehydroalanine and for chloroacetyldehydroalanine (*1*).

TABLE 1.—*Ammonia and pyruvic acid evolved from acetyldehydroalanine in dialyzed extracts of rat tissues*

Tissue [1]	Ammonia [2]	Pyruvic acid [2]	Molar ratio of ammonia to pyruvic acid
Kidney [3]	178	1,278	0.9
Liver [3]	64	462	.9

[1] Extracts of spleen, brain, pancreas, and muscle showed little or no activity.
[2] In terms of micrograms. Theory for ammonia 350, for pyruvic acid 2,200. Substrate at 0.025M concentration.
[3] Results of similar incubation of these tissues with acetyldehydrophenylalanine were completely negative. Tissues dialyzed for 4 hours against distilled water at 5° C.

[1] Received for publication April 1, 1947.
[2] On leave from the Laboratory of Biophysics, University of Brazil, Rio de Janeiro.

It has been taken for granted that enzymatic hydrolysis of the dehydropeptides was effected at the peptide bond, leading to formation of the acyl acid and α-aminoacrylic acid; the latter hydrolyzes spontaneously to yield ammonia and pyruvic acid. Thus:

$$RCONHC(=CH_2)COOH \rightarrow RCOOH + CH_2=C(NH_2)COOH$$
$$CH_2=C(NH_2)COOH \rightarrow NH_3 + CH_3COCOOH \quad (1)$$

The possibility that the peptide may be split to yield the acid amide and pyruvic acid was originally considered by Bergmann and Schleich (*6, 7*), thus:

$$RCONHC(=CH_2)COOH \rightarrow RCONH_2 + CH_3COCOOH \quad (2)$$

This possibility was rejected when Bergmann and Schleich noted that the formation of ammonia from glycine amide was too low to account for the rate of splitting of glycyldehydroalanine. Since the present experimental conditions are different from those employed by Bergmann and Schleich, it was considered desirable to retest the possibility that the dehydropeptides might be split into the corresponding acid amides and pyruvic acid by noting the action of fresh kidney extracts on glycine amide (possible product of glycyldehydroalanine), chloroacetamide (possible product of chloroacetyldehydroalanine), and acetamide (possible product of acetyldehydroalanine). The tests were performed under conditions given for the experiments summarized in table 1.

Substrate	*Ammonia N in micrograms* [1]
Glycine amide	118
Chloroacetamide	0
Acetamide	0

[1] Complete splitting would yield 350 micrograms.

Only glycine amide is split under these conditions, and this to a very much less extent than is glycyldehydroalanine (*1, 5*). In agreement with Bergmann and Schleich, therefore, reaction 1 seems to be the more probable of the two reactions, and the enzymatic hydrolysis of the substrates seems to be effected at the peptide bond. The ratio of nearly one for ammonia to pyruvate is the result of the hydrolysis of this bond, since by reaction 2, chloroacetyldehydroalanine and acetyldehydroalanine would yield pyruvate but no ammonia.

The failure of acetyldehydrophenylalanine to be hydrolyzed under conditions under which acetyldehydroalanine is split demonstrates the effect of the phenyl group in inhibiting the action of the enzyme.

Figure 1 describes the absorption curves in the ultraviolet of glycyldehydroalanine, of chloroacetyldehydroalanine, of acetyldehydroalanine, and of acetyldehydrophenylalanine. The curves at equimolar concentrations of the three aliphatic peptides are very nearly alike. The curves for pyruvic acid and glycylalanine are included for comparison. The absorption of acetyldehydrophenylalanine, except at 2350A, is considerably greater than that of the other dehydropeptides.

Figure 2 describes the changes, with time of incubation, in the absorption of acetyldehydroalanine in the presence of aqueous rat-kidney extracts at pH 8.0. Spectrophotometric determinations were made at time intervals on aliquots of

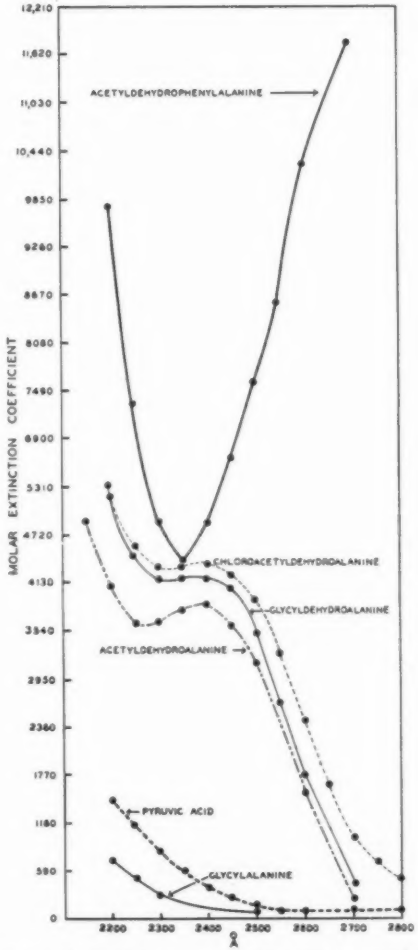

FIGURE 1.—Absorption curves of 1.7 x 10^{-4} M aqueous solutions of dehydropeptides, of pyruvic acid, and of glycylalanine.

0.2 cc. removed from the digests described previously, which were subsequently diluted with 10 cc. of distilled water. The initial concentration of peptide under these conditions is 1.2 x 10^{-4} M. The absorption progressively diminishes with time of incubation, corresponding to a splitting of the substrate. The decrease in absorption at

2500Å after 4 hours of incubation indicates about 60 percent hydrolysis of the substrate. Ammonia determinations made simultaneously are in agreement with this figure.

Dehydropeptidase activity in rat kidney extracts given as before (*5*) in terms of substrate split per hour per milligram of total N in the extract is described in the following tabulation for each of the three substrates tested.

Substrate	*Dehydropeptidase activity* [1]
Glycyldehydroalanine	820
Chloroacetyldehydroalanine	53
Acetyldehydroalanine	13

[1] In terms of milligrams substrate x 10^5 split per hour per milligram total N in aqueous rat-kidney extract. Rate of decrease of absorption measured at 2500Å in Beckman spectrophotometer. pH, 8.0. Concentration of subtrate, 0.025 M.

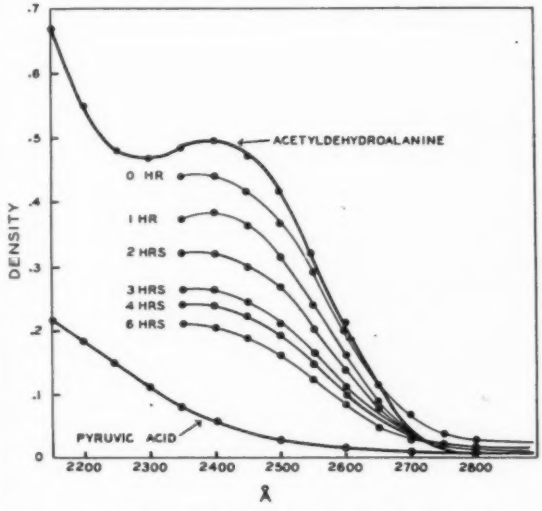

FIGURE 2.—Changes in absorption of acetyldehydroalanine at initial concentration of 1.2 x 10^{-4} M as a result of digestion with rat-kidney extracts. Top curve: substrate in water; bottom curve: pyruvate in water; intermediate curves: digests after various periods of incubation, pH, 8.0.

At pH 6.8, under the conditions given previously (*5*), the activity of rat-liver extract for the three substrates is, respectively; glycyldehydroalanine 86, chloroacetyldehydroalanine 20, and acetyldehydroalanine close to 0. The optimum dehydropeptidase activity is near pH 8.

DISCUSSION

The N-acetyl group is ordinarily one of the most stable linkages known in biologic systems. The detoxication of many primary amines by acetylation is a well-known fact, as illustrated by the behavior of the sulfanilamides. Such compounds as acetylglycine, acetylalanine, and their substituted halogen derivatives like chloroacetylalanine, are nearly completely resistant to enzymatic hydrolysis. Only when an amino group is substituted into the acetyl radical, forming a glycyl residue, is some measure of susceptibility to peptidase action achieved.

The N-acetyl derivative of α-aminoacrylic acid (acetyldehydroalanine) is, however, readily hydrolyzed in aqueous extracts of rat kidney and liver. The N-chloroacetyl derivative is still more readily split, while the N-amino-acetyl (glycyl) derivative is hydrolyzed at an even greater rate under similar conditions. The relative order of susceptibility of the substituted N acetyl peptides is much the same, but the order of magnitude of rate of enzymatic hydrolysis is very much greater in the case of the unsaturated peptides as compared with their saturated homologues (*1*). The failure of acetyldehydrophenylalanine to be hydrolyzed under conditions under which acetyldehydroalanine is readily split, suggests that the presence of the phenyl group in the former compound is inhibitory to the action of the enzyme. This is interesting in view of the fact that Bergmann and Schleich (*6, 7*) noted that glycyldehydrophenylalanine was as readily hydrolyzed in kidney extracts as glycyldehydroalanine. Exact kinetic studies employing these two glycyl dehydropeptides would be helpful in determining the nature of the influence of the phenyl group in this class of substrates.

Acetyldehydroalanine, like glycyldehydroalanine and chloroacetyldehydroalanine, possesses a characteristic absorption in the ultraviolet region of the spectrum, and, according to the suggestion advanced earlier, probably exists in at least the following tautomeric equilibrium (*5*):

$$CH_3CONHC(=CH_2)COOH \rightleftarrows CH_3CON=C(CH_3)COOH \quad (3)$$

As the hydrolysis of this compound proceeds, the characteristic absorption decreases. The measurement of this rate of decrease furnishes an alternative method of observing dehydropeptidase activity in addition to that of measuring the ammonia and pyruvic acid produced (*5*).

The very considerable absorption in the ultraviolet of acetyldehydrophenylalanine is most probably attributable to an enhancement of the characteristic absorption of the benzene ring by the adjacent aliphatic double bond.

SUMMARY

The enzymatic hydrolysis of acetyldehydroalanine in aqueous extracts of rat kidney and liver has been observed by methods involving measurement of the amount of ammonia and pyruvic acid produced, and by spectrophotometric measure-

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ments in the ultraviolet of the rate of change in the characteristic absorption of this substrate. The molar ratio of ammonia to pyruvate produced from the substrate in extracts of either tissue is 0.9, or not far from unity, indicating that the acetyldehydroalanine is split at the peptide bond. This is further supported by the fact that acetamide is not split in extracts of either tissue, and hence the peptide could not be hydrolyzed to amide and pyruvate.

Acetyldehydrophenylalanine is not split under the conditions employed.

REFERENCES

(*1*) Greenstein, J. P., and Leuthardt, F. M.: Degradation of cystine peptides by tissues. I. Exocystine desulfurase and dehydropeptidase in rat-liver extracts. J. Nat. Cancer Inst. 5: 209–221 (1944).

(*2*) ——— and Leuthardt, F. M.: Degradation of cystine peptides by tissues. II. Distribution of exocystine desulfurase and dehydropeptidase in tissue extracts of various species. J. Nat. Cancer Inst. 5: 223–225 (1944).

(*3*) ——— and Leuthardt, F. M.: Degradation of cystine peptides by tissues. III. Absence of exocystine desulfurase and dehydropeptidase in tumors. J. Nat. Cancer Inst. 5: 249 (1945).

(*4*) ——— and Leuthardt, F. M.: Degradation of cystine peptides by tissues. IV. Dehydropeptidase activity in normal and neoplastic tissues. J. Nat. Cancer Inst. 6: 197–201 (1946).

(*5*) Carter, C. E., and Greenstein, J. P.: Spectrophotometric determination of dehydropeptidase activity in normal and neoplastic tissues. J. Nat. Cancer Inst. 7: 51–56 (1946).

(*6*) Bergmann, M., and Schleich, H.: Über die enzymatische Spaltung dehydrierter Peptide. Auffindung einer Dehydropeptidase. Ztschr. f. physiol. Chem. 205: 65–75 (1932).

(*7*) Bergmann, M., and Schleich, H.: Weiteres über Dehydro-dipeptidase. Über die enzymatische Angreifbarkeit von Verbindungen aus Brenztraubensäure und Aminosäure. Ztschr. f. physiol. Chem. 207: 235–240 (1932).

(*8*) Bergmann, M., and Grafe, K.: Synthese eines Peptids und andere Abkommlinge der α-Aminoacrylsäure (Dehydroalanine) aus Brenztraubensäure. Ztschr. f. physiol. Chem. 187: 187–195 (1930).

(*9*) Greenstein, J. P., and Carter, C. E.: Influence of α-keto acids on the desamidation of amino acid amides. J. Nat. Cancer Inst. 7: 57–60 (1946).

STUDIES ON THE AGENT OF CHICKEN TUMOR I. I. THE RELATIVE POTENCIES OF SERIAL EXTRACTS AND THEIR COMPARATIVE VALUES AS SOURCE MATERIALS FOR PURIFICATION STUDIES[1]

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INTRODUCTION

The high content of mucoid[2] substances in extracts of chicken-tumor-I (*1*) tissue is a complicating factor in attempts to purify the active tumor-producing agent by any of the methods generally employed for virus purification, e. g., differential centrifugation, differential adsorption, precipitation by chemical substances, and filtration. A means was therefore sought for obtaining the active agent free from this mucoid material, insofar as possible. The studies of Murphy and Sturm (*2*) on the serial extraction of desiccated chicken-tumor-I tissue suggested the possibility that fresh tumor tissue (which is more potent in tumor-producing activity than desiccated tissue) might be extracted serially without too rapid a reduction of potency in the successive extracts, and that most of the mucoid material (considered to be primarily extracellular) might be washed away in the first extracts, leaving some later extracts relatively free of this substance but still containing sufficient tumor agent for practical experimental purposes. The present studies were made to determine the relative tumor-producing potencies of serial extracts of fresh tumor tissue, prepared with 0.9 percent sodium chloride solution. In addition, determinations were made of the total nitrogen content, of the potency-nitrogen ratio, and, in some instances, of the hydrogen-ion concentration.

MATERIALS AND METHODS

The tumor tissue was from tumors produced in the wings of young (6 to 8 weeks old) chickens by cell transplantation. Tumors were removed on the seventh or eighth day following transplantation, and only soft, healthy tissue, which could readily be scooped out with a sterile spoon, was taken. It was stored in a dry-ice cabinet for several weeks prior to use in the present experiments.

The test chickens were of the New Hampshire Red breed and were hatched and raised in this laboratory. They were 3 to 4 weeks old at time of inoculation.

Total nitrogen determinations were made by the direct nesslerization micro-Kjeldahl method of Koch and McMeekin (*3*) using an Aminco Type F photometer for the color comparisons. The volume of samples used in tests varied from 0.1 to 10 ml., as required, depending upon the level of nitrogen concentration.

A Beckman glass-electrode pH meter was used for hydrogen-ion determinations.

Bioassays were carried out with a balanced experimental design patterned after that described by Bliss and Marks (*4, 5*) for the assay of insulin. The biological response used for measuring activity was the reciprocal of the latent period ($\times$ 100) previously described (*6, 7*). A 4-x-4 Latin square, in which the columns were represented by chickens and the rows by sites (both wings and both breast regions), or vice versa, constituted the basic design. In each experiment one of the tumor-agent preparations was arbitrarily designated as "standard" and used as a reference material for determining the relative potency of each of the other preparations of the same experiment, in a separate 4-dose (2 doses of standard and 2 of unknown) assay experiment. The dosages are expressed in terms of the logarithm of the number of grams of tumor tissue extracted. Similar doses were employed for both standard and unknown, and there was a tenfold difference between the 2 doses of the same material. The doses used in assay determinations were chosen, on a basis of preliminary determinations, so that the responses would fall within the range in which the relationship between response and the logarithm of dose was linear. (See Bryan (*6*).) A Latin-square group with 10 chickens per block was used for each potency estimate, giving a total of 40 unit observations for each dose of each material. In computing the potency ratios and their errors, a common estimate of slope and of slope variance was employed for all simultaneous assays within the same experiment.

The preparation of materials and the details pertaining to individual experiments are described in subsequent sections.

EXPERIMENTAL PROCEDURE AND RESULTS

Experiment 1

Experiment 1 was done to compare the relative potencies of six successive extracts of the same batch (10 gm.) of tumor tissue prepared with 0.9 percent NaCl solution.[3] In each extraction, the tumor tissue was placed in a Waring blender with

[1] Received for publication April 1, 1947.

[2] This term is used to designate substances that impart a slimy quality to chicken-tumor extracts and is not intended to imply known chemical composition.

[3] Hereinafter referred to as NaCl solution.

100 ml. of NaCl solution and blended for a period of 1 minute; 200 ml. of NaCl solution was then added, and the mixture was again blended for 1 minute. The homogenized suspension was centrifuged for 20 minutes at 4,000 r. p. m. in an angle head of an ordinary centrifuge [4] to remove gross tissue particles. The supernatant fluid was carefully pipetted off, except for small amounts left above the firmly packed pellets to prevent their disturbance. The centrifuge tubes (100-ml. capacity) containing the pellets were inverted and allowed to drain, after which the sedimented tissue was recovered from all tubes and used for the next extraction. The supernatant fluids obtained in the successive extractions were designated serially as extracts 1 through 6.

The first extract was used as a standard of reference in assaying the other materials (i. e., extracts 2 through 6), and all potencies are expressed in relation to the potency of the first extract, which was considered to be unity, or 100 percent. Analyses of variance of the biological responses are given in table 1.[5] Figure 1 shows the mean responses to standard and unknown materials of the five separate assay determinations, plotted against logarithm of dosage. The regression lines all have the same slope (the common slope, b_c, computed from all data of experiment 1) and are drawn through the respective coordinate points, mean log dose ($\bar{x}$), and mean response ($\bar{y}$), shown on the graphs as small closed circles. The horizontal interval between each pair of parallel lines (or the vertical distance divided by the slope constant, b_c), when measured on the abscissa scale, gives the logarithm of the potency ratio between the paired materials. The algebraically derived log ratios and their standard errors are given in table 2 together with the corresponding antilogs ($\times$ 100) which represent the actual potency ratios in percent. The nitrogen and pH determinations and the relative values for nitrogen, potency, and potency-nitrogen ratios are summarized in table 3. The relative values are charted for extracts 1 through 4 in figure 2; the values for extracts 5 and 6 were too small to be shown on the graph.

[4] International centrifuge No. 2; head No. 845.

[5] The departure from parallelism of the two dose-response curves was statistically significant in data of the third assay group of table 1. (See also plotted points in middle chart of figure 1.) This result is occasionally obtained under the conditions of the present experiments and indicates that the dosage interval employed (i. e. $\times$ 10) is not adequate to give a satisfactory estimate of slope in the separate assay groups under the other conditions of the experiment, namely the relatively low regression of response on dose (the dose-effect curves show the regression on log dose), the relatively wide limits of experimental error, and only 40 unit observations per dose. The slope of the dose-effect curve and the magnitude of the experimental error are inherent in the materials and methods employed and cannot be altered readily. Satisfactory estimates of slope might be obtained with 40 observations per dose if the doses could be spaced widely enough apart, but as already pointed out (6), the chickens die from tumors induced by the higher doses before the lower ones have had time to show their effects when the interval between doses is too great. A dosage interval of $\times$ 10, for the two samples of the same material, has been found to be most satisfactory for routine assays: this interval cannot be appreciably increased (e. g., to $\times$ 100) unless the difference in potency between the two materials being compared (e. g., standard, and unknown) is known in advance to be less than tenfold.

Since, in addition to the slope and the experimental error, the dosage interval is also fixed for the present materials and methods, the only remaining way of improving the slope estimate is to increase the number of unit observations per dose group. Thus, if only two materials were to be compared in a single assay determination of the present type, it would be necessary to use $5 \times 40 = 200$ unit observations per dose group in order to obtain an estimate of slope as accurate as the common slope of the present experiment which is based upon all data of the 5 simultaneous assays.

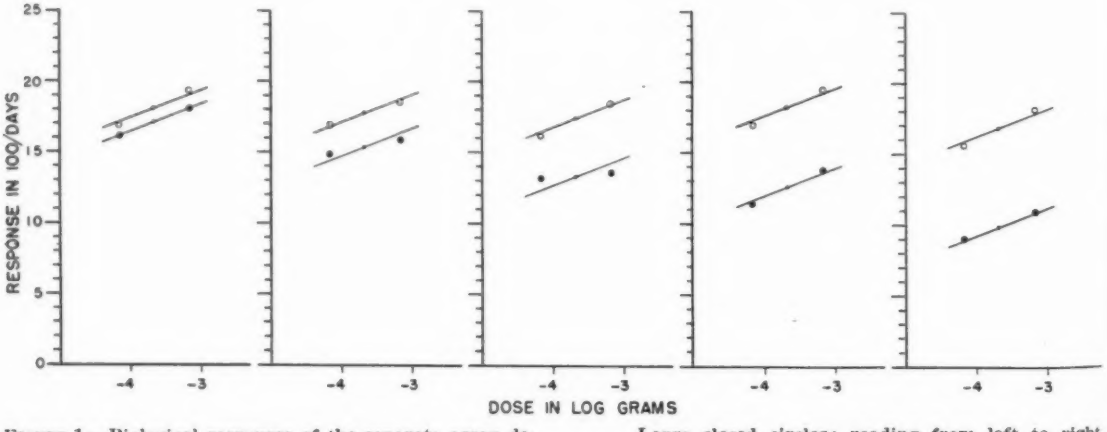

FIGURE 1.—Biological responses of the separate assay determinations of experiment 1. See text.

Open circles: extract 1; the common "standard" for all assay groups.

Large closed circles: reading from left to right, extracts 2 through 6 ("unknowns").

The dose-response curves have a common slope and are drawn through the means (small closed circles) for the respective materials.

TABLE 1.—*Analyses of variance of biological responses of the separate assay groups of experiment 1*

TABLE 1.—*Analyses of variance of biological responses of the separate assay groups of experiment 1*

Variation	Bioassay determinations																					
	1 (S=Extract 1; U=Extract 2)[1]				2 (S=Extract 1; U=Extract 3)[1]				3 (S=Extract 1; U=Extract 4)[1]				4 (S=Extract 1; U=Extract 5)[1]				5 (S=Extract 1; U=Extract 6)[1]			
	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F
Differences between chickens	751.0228	39	19.257	[2] 5.639	1,567.0740	39	40.181	[2] 10.283	2,323.8844	39	59.587	[2] 10.909	1,527.1994	39	39.159	[2] 2.807	1,126.9000	39	28.895	[2] 1.960
Differences between sites	224.3953	3	74.798	[2] 21.905	200.9445	3	66.982	[2] 17.089	157.3757	3	52.459	[2] 9.604	138.3217	3	46.107	[2] 3.306	171.2275	3	57.076	[2] 3.932
Differences between doses	262.7413	3	87.580	[2] 25.648	292.3630	3	97.454	[2] 24.747	722.1921	3	240.731	[2] 44.071	1,494.6637	3	498.221	[2] 35.720	2,171.1425	3	723.714	[2] 49.853
Differences between samples	[3] (48.4000)	[3] (1)	48.400	[2] 14.174	[3] (221.8410)	[3] (1)	221.841	[2] 57.332	[3] (626.0766)	[3] (1)	626.077	[2] 114.618	[3](1,263.9381)	[3] (1)	1,263.983	[2] 90.617	[3](1,934.8810)	[3] (1)	1,934.881	[2] 133.263
Slope of dose effect curve	[3] (210.6810)	[3] (1)	210.681	[2] 61.698	[3] (68.1210)	[3] (1)	68.121	[2] 17.598	[3] (67.4701)	[3] (1)	67.470	[2] 12.352	[3] (230.6401)	[3] (1)	230.640	[2] 16.536	[3] (204.7562)	[3] (1)	204.756	[2] 14.105
Departure from parallelism	[3] (3.6557)	[3] (1)	3.6557	[4] 1.071	[3] (2.4010)	[3] (1)	2.4010	[4] .610	[3] (28.6456)	[3] (1)	28.646	[2] 5.244	[3] (.0856)	[3] (1)	.0856	[4] .0061	[3] (4.6240)	[3] (1)	4.6240	[4] .318
Experimental error	389.2784	114	3.4147	.....	448.9425	114	3.9381	.....	622.7047	114	5.4623	.....	1,590.0871	114	13.9481	.....	1,654.9450	114	14.5171	.....
Total	1,627.4378	159	.....	.....	2,509.3240	159	.....	.....	3,826.1569	159	.....	.....	4,750.2719	159	.....	.....	5,124.2150	159	.....	.....
Correction	49,470.1222	1	.....	.....	43,612.8160	1	.....	.....	37,524.8131	1	.....	.....	38,492.7181	1	.....	.....	28,890.6256	1	.....	.....

[1] S=standard; U=unknown.
[2] Significant at 1 percent level.
[3] Significant at 5 percent level.
[4] Not significant.
[5] Values in parentheses are subvalues under those for differences between doses, to which they sum, and are not reentered in deriving the grand totals.

FIGURE 2.—Relative results of experiment 1. See table 3 and text.
Cross-hatched columns: nitrogen;
Solid columns: potency;
Dotted columns: potency-nitrogen ratio.

TABLE 2.—*Summary of bioassay determinations of experiment 1*

Assay	Standard	Unknown	Logarithm of potency ratio (M)	Standard error of M (s_M)	Potency ratio in percent (antilog of M) (×100)	Standard error of potency ratio [1]
1	Extract 1	Extract 2	−0.5752	±0.1619	26.60	+12.66 −8.58
2	do	Extract 3	−1.2314	±.2466	5.87	+4.49 −2.55
3	do	Extract 4	−2.0686	±.4182	.854	+1.486 −.534
4	do	Extract 5	−2.9392	±.9003	.115	+.795 −.105
5	do	Extract 6	−3.6366	±1.1145	.023	+.277 −.021

[1] Derived by difference from the antilogs (× 100) of the limiting log values corresponding to $M+s_M$ and $M-s_M$; apply to values of preceding column.

TABLE 3.—*Summary of results of experiment 1* [1]

Determination	Extract					
	1	2	3	4	5	6
Nitrogen, milligrams per milliliter	0.30	0.005	0.003	0.001	0.0006	0.0008
Nitrogen, percent	100.0	1.67	1.00	.333	.200	.266
Potency, do	100.0	26.6	5.87	.854	.115	.023
Potency-nitrogen ratio, percent potency / percent nitrogen	1.0	15.96	5.87	2.56	.575	.086
pH	6.3	5.8	5.9	6.0	5.9	6.1

[1] In determining the relative values the results on extract 1 were considered as 100 percent.

The differences in potency between standard and unknown materials were highly significant in all assays (table 1), and the general trends shown in tables 2 and 3 and in figure 2 are real; however, true numerical values have been estimated only

within certain limits as indicated by the standard errors of table 2. The degree of variability shown is that which is inherent in the present test materials and methods when only 40 unit observations are used for each dose. Only the most probable potency values were used in computing the potency-nitrogen ratios and in comparing the results of different extracts. The results are sufficiently accurate for the essential purposes of this experiment. If greater accuracy had been required, it would have been necessary to increase the number of unit biological observations accordingly.

Figure 2 shows that both the nitrogen and the potency dropped rapidly in successive extracts (the values were negligible and too low to be charted in the last two extracts). The greatest differences were between extracts 1 and 2. This is in contrast with the findings of Murphy and Sturm (*2*) on the serial extraction of desiccated chicken-tumor-I tissues with distilled water; these investigators observed a progressive increase rather than a decrease in potency in the first 4 serial extracts accompanied by a progressive decrease in nitrogen similar to that shown here. In the present instance, both nitrogen and potency decreased with successive extractions; but the nitrogen showed a relatively greater drop, and the ratio of potency to nitrogen was higher in subsequent extracts than in the first. The highest ratio was observed with extract 2, in which case it was about 16 times the ratio for extract 1. This indicates that the second extract is a much purer product than the first, with respect to the absence of nonactive nitrogenous material. In addition, the second extract was grossly free of the mucoid substances which give first extracts a viscous quality and which complicate further purification procedures when first extracts are used.

The bioassay results showed extract 2 to be only about one-fourth as potent as extract 1 (considering the most probable value of the potency estimate), but this difference is not very serious and could readily be compensated for under practical laboratory conditions, if necessary. The increased purity of the second extract and its relative freedom from mucoid substances far outweigh the loss in potency; and it is suggested that second extracts constitute a more desirable starting material for purification of the agent of chicken tumor I than first extracts, which have been generally employed. The low potency of extracts subsequent to the second and the fact that they showed no advantage with respect to potency-nitrogen ratio indicate that they are less desirable sources of tumor agent than the second extract.

Experiment 2

In the second experiment several materials were intercompared, but only two of them and the standard reference material with which they were compared are considered in this report.[6] The experimental materials were a first and second extract of fresh tumor tissue prepared with NaCl solution in exactly the same manner as described for experiment 1. The standard reference material with which each was compared (in a separate, balanced, bioassay determination) was an ultracentrifuge concentrate of a second extract resuspended in phosphate buffer (pH 7.0; 0.005M). Analyses of variance of the biological responses are given in table 4, and the mean responses are plotted against logarithm of dose in figure 3; the bioassay results are summarized in table 5. In order that the results of experiment 2 could be compared directly

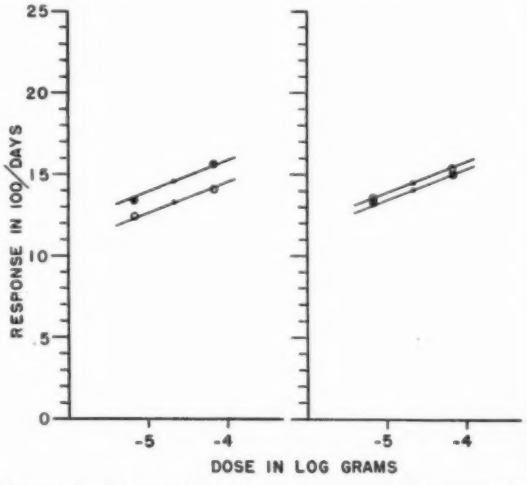

Figure 3.—Biological responses of the separate assay determinations of experiment 2. See text.
Open circles: Ultracentrifuge concentrate; the common "standard" for both assay groups.
Large closed circles: reading from left to right, extract 1 and extract 2 ("unknowns").
The dose-response curves have a common slope and are drawn through the means (small closed circles) for the respective materials.

with those of experiment 1, the relative values for nitrogen and potency and the potency-nitrogen ratios were determined in the same manner as in experiment 1. For this purpose, the nitrogen content of the first extract and the relative potency derived in the bioassay of the first extract were each taken as 100 percent, and the relative values for the second extract were computed accordingly. The results are shown in table 6 and in figure 4. The interpretations of the tables and figures of ex-

[6] The common slope for bioassay determinations was determined from all materials of the experiment.

TABLE 4.—*Analyses of variance of biological responses of the separate assay groups of experiment 2*

Variation	Bioassay determinations							
	1 (S=U. C. concentrate; U=Extract 1) [1]				2 (S=U. C. concentrate; U=Extract 2) [1]			
	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F
Differences between chickens	1,897.3984	39	48.651	[2] 10.68	313.8948	39	8.049	[2] 5.155
Difference between sites	260.3781	3	86.793	[2] 19.06	180.4453	3	60.148	[2] 38.522
Differences between doses	229.5457	3	76.515	[2] 16.80	134.2433	3	44.748	[2] 28.659
Differences between samples	[3] (60.1476)	[3] (1)	60.148	[2] 13.20	[3] (4.9000)	[3] (1)	4.900	[4] 3.138
Slope of dose effect curve	[3] (166.6681)	[3] (1)	166.668	[2] 36.59	[3] (128.881)	[3] (1)	128.881	[2] 82.542
Departure from parallelism	[3] (2.7301)	[3] (1)	2.730	[4] .60	[3] (.4622)	[3] (1)	.462	[4] .296
Experimental error	519.2537	114	4.548		177.9964	114	1.561	
Total	2,906.5759	159			806.58	159		
Correction	30,871.9141	1			32,724.12	1		

[1] S=standard; U=unknown.
[2] Significant at 1 percent level.
[3] Values in parentheses are subvalues under those for differences between doses, to which they sum, and are not reentered in deriving the grand totals.
[4] Not significant.

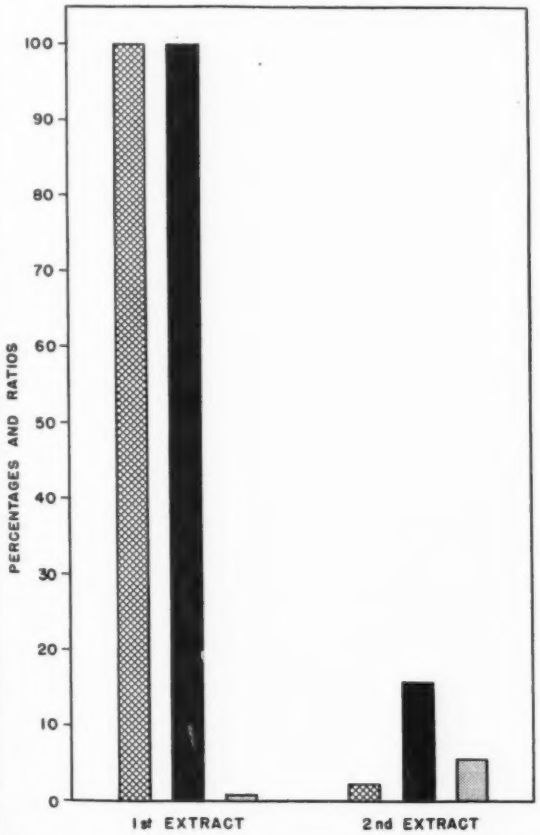

FIGURE 4.—Relative results of experiment 2. See table 6 and text.
Cross-hatched columns: nitrogen;
Solid columns: potency;
Dotted columns: potency-nitrogen ratio.

periment 2 are similar to those described in experiment 1.

TABLE 5.—*Summary of bioassay determinations of experiment 2*

Assay	Standard	Unknown	Logarithm of potency ratio (M)	Standard error of M (s_M)	Potency ratio in percent (antilog of M) (×100)	Standard error of potency ratio [1]
1	U. C. concentrate.	Extract 1	0.62861	±0.20369	425.22	{+254.46 −159.20
2	do	Extract 2	−.17941	±.10270	66.16	{+17.65 −13.93

[1] See footnote 1 of table 2.

TABLE 6.—*Summary of results of experiment 2*

Determination	Extract 1	Extract 2
Nitrogen, milligrams per milliliter	0.30	0.008
Nitrogen, percent [1]	100.0	2.67
Potency, percent, relative to common standard	425.216	66.159
Potency, percent, relative to extract 1 [1]	100.0	15.56
Potency-nitrogen ratio, percent nitrogen / percent potency [1]	1.0	5.83

[1] Percentage values based upon results of extract 1 as 100 percent.

In experiment 2, the potency of the second extract was only about one-sixth that of the first extract, whereas in experiment 1 it was about one-fourth. These two results do not differ more than experimental error, however, and the findings in the two experiments are entirely consistent within limits of chance variation. The conclusion suggested by experiment 1, namely, that second extracts are sufficiently potent for practical use and may constitute better sources of agent (for studies on purification) than first extracts is therefore substantiated.

Additional Data on the Comparison of First and Second Serial Extracts

Second extracts are now being employed routinely in this laboratory as sources of tumor agent. In several instances, the first extracts have been tested and comparisons made between the relative potencies of first and second serial extracts prepared from the same batch of tumor tissue. Three additional quantitative experiments and one semiquantitative experiment of this type have been completed, and the data have been analyzed statistically. The final results are included herewith (table 7) to provide further evidence in support of the conclusions drawn from the foregoing experiments.

Table 7 shows a summary of the results of six experiments in which first and second extracts were compared. The first two represent experiments 1 and 2 already described; experiments 3 through 6 represent the additional observations. In experiment 3, the technical conditions and the method of preparation of extracts were exactly the same as those employed in experiments 1 and 2. (See above.) Experiment 4 differed only in the manner in which the tissue extracts were cleared of tissue debris and aggregated colloid particles. Both the first and the second extracts were cleared by three consecutive centrifuge runs as follows: (1) In an angle head of an ordinary centrifuge as in experiments 1 and 2, above; (2) in a multispeed centrifuge [7] spinning at approximately 10,000 r. p. m. (5,800 g) for 10 minutes; and (3) in the same centrifuge [7] at full speed (18,000 g) for 1 minute. After each clearance run, the middle fraction of supernatent fluid was taken, and small amounts at the top of the centrifuge tube and just above the sediment were discarded. The technique employed in experiments 5 and 6 differed from the others in the following respects: (1) The volume of NaCl solution in which the tissue was blended for the second extraction was 200 ml., instead of 300 ml.; and (2) both the first and the second extracts were cleared of tissue debris and aggregated material by two consecutive runs-up-and-down (i. e., 18,000 g for 1 minute) in a multispeed centrifuge.

Table 7.—*Summary of results of six experiments on the comparison of first and second serial extracts*

Experiment No.	First extracts					Second extracts				
	Volume of NaCl solution in which homogenized	Potency	Nitrogen		Ratio percent potency / percent nitrogen	Volume of NaCl solution in which homogenized	Potency	Nitrogen		Ratio percent potency / percent nitrogen
			Milligrams per milliliter	Percent				Milligrams per milliliter	Percent	
	Ml.	*Pct.*				*Ml.*	*Pct.*			
1	300	100	0.30	100	1.0	300	28.6	0.005	1.67	15.96
2	300	100	.30	100	1.0	300	15.6	.008	2.67	5.83
3	300	100	.221	100	1.0	300	20.9	.006	2.76	7.57
4	300	100	.132	100	1.0	300	4.17	.0017	1.36	3.07
5	300	100	.145	100	1.0	200	[1] (100)	.0046	3.17	
6	300	100	.114	100	1.0	200	70.1	.0063	5.51	12.73

[1] Semiquantitative estimate only.

The results of experiment 3 showed that the relative potency as well as the potency-nitrogen ratio of the second extract was intermediate between the corresponding values for the first two experiments and was consistent with them within limits of chance variation. The results of experiment 4 differed significantly from the first three experiments with respect to total nitrogen in the first extract and to both nitrogen and potency in the second extract, probably as a result of the excessive clearance treatment. (See above.) Although the potency of the second extract was only about 1/25 that of the first extract in this case, the second extract was nevertheless highly active biologically since the 1:10 dilution of it produced tumors in 6.5 days on an average.

The second extracts of experiments 5 and 6 were considerably more potent (relative to their respective first extracts) than those of preceding experiments. Whether this is a fortuitous result due to variations between batches of tumor tissue, or whether the increase in potency was due to a more efficient breaking up of tumor cells, and release of agent, when a smaller volume of NaCl solution was used for homogenization cannot be definitely determined from present data; but it is not unlikely that more efficient homogenization procedures than those described here may be found by varying either the volume of solution, the time of blending in the Waring blender, or both.

These additional data confirm the superiority of second over first extracts as sources of tumor agent for studies on purification and suggest changes in technique, which might be expected to result in greater yields of active tumor agent than

[7] Belt-driven multispeed attachment of an International Centrifuge No. 2; head No. 295.

were found under conditions described in preceding sections.

SUMMARY

The relative potencies and total nitrogen contents of serial extracts of fresh chicken-tumor-I tissue, prepared with 0.9 percent NaCl solution, were studied in experiments carried out with quantitative biological techniques. The results showed that second extracts contained considerably less mucoid and nonactive nitrogenous material than first extracts and were sufficiently potent to serve as practical sources of tumor agent for experimental purposes.

Serial extracts subsequent to the second were considerably weaker in tumor-producing potency and offered no advantage, with respect to purity, over second extracts.

It is suggested that second extracts may constitute a more desirable starting material for studies on purification of the active agent than first extracts which have been generally employed.

REFERENCES

(1) Rous, P.: A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exper. Med. 13: 397 (1911).

(2) Murphy, J. B., and Sturm, E.: Properties of the causative agent of a chicken tumor IV. Association of an inhibitor with the active principle. J. Exper. Med. 56: 107–116 (1932).

(3) Koch, F. C., and McMeekin, T. L. New direct nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. J. Am. Chem. Soc. 56: 2066–2069 (1924).

(4) Bliss, C. I., and Marks, H. P.: The biological assay of insulin. I. Some general considerations directed to increasing the precision of the curve relating dosage and graded response. Quart. J. Pharm. & Pharmacol. 12: 82–110 (1939).

(5) Bliss, C. I., and Marks, H. P.: The biological assay of insulin. II. The estimation of drug potency from a graded response. Quart. J. Pharm. & Pharmacol. 12: 182–205 (1939).

(6) Bryan, W. R.: Quantitative studies on the latent period of tumors induced with subcutaneous injections of the agent of chicken tumor I. I. Curve relating dosage of agent and chicken response. J. Nat. Cancer Inst. 6: 225–237 (1946).

(7) ———: Quantitative studies on the latent period of tumors induced with subcutaneous injections of the agent of chicken tumor I. II. Estimation of the latent period. J. Nat. Cancer Inst. 6: 373–377 (1946).

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EFFECT OF CASTRATION AND SEX HORMONES ON THE INDUCTION OF TUMORS IN MICE WITH *o*-AMINOAZOTOLUENE [1]

By HOWARD B. ANDERVONT, *biologist*, and THELMA B. DUNN, *pathologist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Subcutaneous administration of 3, 4, 5, 6-dibenzcarbazole (*1*) or *o*-aminoazotoluene (*2*) to mice revealed a pronounced sex variation in susceptibility to induced hepatic changes and hepatomas. The investigations reported here were designed to study further the effects of sex on susceptibility to *o*-aminoazotoluene by producing changes in the hormonal condition of the animals. In one experiment the effects of castration were studied while in a second the effects of castration alone and castration followed by administration of testosterone propionate were ascertained.

MATERIALS AND METHODS

Mice of strain C (*3*) were used because they were known to exhibit a pronounced sex difference in susceptibility to *o*-aminoazotoluene and could also be used to determine the occurrence of induced pulmonary tumors and hemangio-endotheliomas (*2*). All were raised in this laboratory. They were maintained under uniform environmental conditions, fed Purina laboratory chow pellets, and given an unlimited supply of drinking water. Males and females were kept in separate cages, but the intact and the castrate animals were not segregated. They were housed in wooden boxes, 28 cm. long, 18 cm. wide, and 15 cm. deep, with 8 mice to a box.

Necropsies were performed on all mice. Those that had received an implanted pellet were examined for the presence of the pellet. The degree of gross hepatic reaction (nodular surface) was noted and a piece of liver prepared for histologic study. When hepatomas were present, the liver specimen included both tumor and liver tissue. The gross and microscopic hepatic changes have been described in earlier papers (*2, 4*). The lungs were examined for the presence and number of macroscopic tumors. A kidney section was obtained from most of the animals. All tissues were fixed in Tellyesniczky's fluid (2 parts formol, 20 parts 70 percent alcohol, and 1 part acetic acid) and stained with hematoxylin and eosin.

The *o*-aminoazotoluene was procured from the Eastman Kodak Co. and was used without further purification. It was moistened with glycerol (6 drops from a 1-cc. pipette to 200 mg.) and injected subcutaneously by means of a trocar. Each mouse received approximately 10 mg. at each injection.

[1] Received for publication April 1, 1947.

EXPERIMENT 1

This experiment was performed to ascertain the effect of castration upon the susceptibility of both sexes to induced hepatic lesions. The mice were castrated when 4 to 6 weeks of age and received the first injection of *o*-aminoazotoluene 2 weeks later, when all were 2 to 3 months old. The compound, totaling approximately 70 mg. to each mouse, was injected seven times at monthly intervals. It was administered dorsally at the base of the tail to all animals.

The occurrence of gross hepatic reactions is summarized in table 1, and the presence of hepatic and pulmonary tumors is presented in table 2. As in earlier work, the intact females were more susceptible to the induction of hepatic changes and hepatomas than were the intact males. Castration of males increased their susceptibility to the induction of both hepatic changes and hepatomas. This finding was similar to the results of an earlier experiment (*1*) in which castration increased the susceptibility of male mice to the occurrence of hepatic changes following the subcutaneous in-

TABLE 1.—*Induction of macroscopic hepatic changes in intact and castrate strain C mice following subcutaneous administration of 70 mg. of o-aminoazotoluene*

[Numerator = Mice killed
Denominator = Mice with hepatic changes]

Group	Number of mice	Time after first injection (in months)					Total	Incidence
		6	7	8	9	10		
								Pct.
Intact females	7	-----	-----	1/1	3/3	3/3	7/7	100
Castrate females	38	-----	-----	13/8	15/14	10/8	38/30	79
Intact males	7	-----	-----	1/0	4/0	2/1	7/1	14
Castrate males	42	2/1	1/1	8/6	16/16	15/14	42/38	90

TABLE 2.—*Induction of pulmonary tumors and hepatic tumors in intact and castrate strain C mice following subcutaneous administration of 70 mg. of o-aminoazotoluene*

[Numerator = mice with pulmonary tumors
Denominator = mice with hepatic tumors]

Group	Number of mice	Time after first injection (in months)					Total	Incidence
		6	7	8	9	10		
								Pct.
Intact females	7	-----	-----	0/1	2/2	3/2	5/5	71/71
Castrate females	38	-----	-----	6/2	8/8	10/6	24/16	63/43
Intact males	7	-----	-----	1/0	2/0	2/1	5/1	71/14
Castrate males	42	-----	-----	3/2	6/7	12/6	21/15	50/36

[1] See table 1 for number of mice autopsied each month.

jection of 3,4,5,6-dibenzcarbazole. There was evidence suggesting that castrate females were more resistant than intact females to induced hepatic changes and hepatomas.

The most interesting outcome of the experiment was the appearance of hemangio-endotheliomas in the experimental animals. These were histologically similar to those described previously (*2*) and likewise showed considerable histologic variation. Some were made up of numerous dilated vascular channels lined by endothelial cells which appeared to be well differentiated, while others consisted of compact areas of large oval or spindle cells, with frequent mitoses. These tumors generally were surrounded by a wide hemorrhagic zone, in which no neoplastic cells were found and which appeared to be a reaction in the surrounding normal tissue to the neoplasm. This zone in the lungs consisted of blood-filled alveoli, and in subcutaneous areas there appeared to be an extreme dilation of vessels and extravasation of erythrocytes. This peripheral reaction was remarkable and often confusing, since the central mass of neoplastic cells was sometimes missed in sectioning. These tumors are discussed later.

Table 2 also indicates that, as in previous investigations (*1*), the carcinogen induced pulmonary tumors in mice of strain C.

Experiment 2

The production of hepatic reactions in all intact females of experiment 1 by the administration of 70 mg. of *o*-aminoazotoluene, together with the increased susceptibility of the castrate males and suggested increased resistance of the castrate females, served as a basis for further investigation.

The experiment was designed to ascertain the effect of testosterone propionate upon the induction of hepatic lesions in castrate animals of both sexes. The mice were born between August 3 and September 7, 1945, and when possible, each litter was divided so that each contained an intact, castrate, and castrate plus testosterone representative of each sex.

Castration was done when the animals were 1 month old, and a pellet [2] of 50 percent testosterone propionate in cholesterol (*5*) weighing approximately 3 mg. was inserted subcutaneously into the left flank of the castrates on November 1, 1945. Each mouse received four successive monthly injections of *o*-aminoazotoluene in the right axilla for a total of approximately 40 mg. The first injection was given on November 15, 1945.

When an animal died or was killed, its litter mates of the same sex were also killed.

The results of macroscopic examinations for hepatic reactions are listed in table 3. Intact females were again more susceptible to the lesion than were their male litter mates. Microscopic changes in the liver and the hepatomas which developed were essentially similar to those already described (*2*). In the liver sections examined in the present experiment, a considerable difference in the size of the cells in the vicinity of the central

[2] Obtained from Dr. M. B. Shimkin, of the National Cancer Institute.

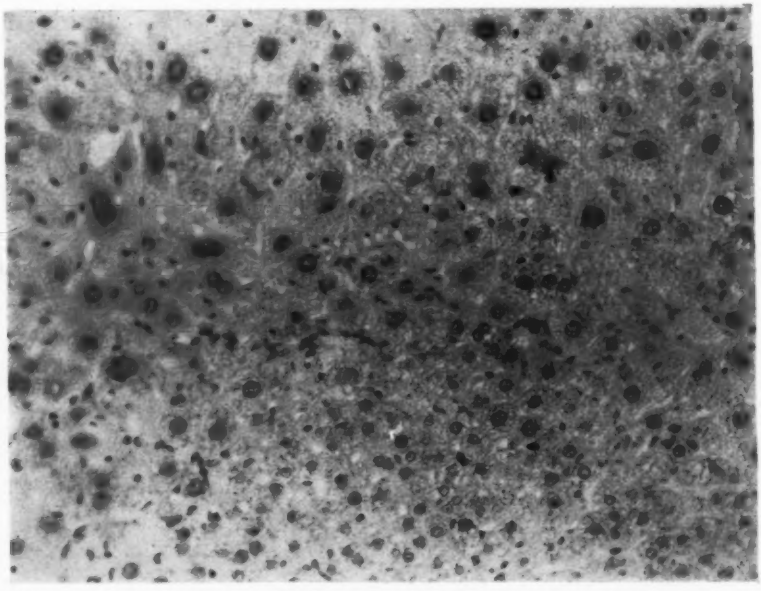

Figure 1.—Variations in size of liver cells, showing a zonal distribution. Hematoxylin and eosin stain. × 235.

vein and those near the portal vessels was frequently observed (fig. 1). The cells in the region of the central veins were greatly enlarged, and the swollen nuclei often contained one or more bodies (fig. 2). These intranuclear bodies are of interest. They have been previously described by Findlay (*6*) and by Andrew et al. (*7*). Findlay observed them in the Claxton strain of mice and noted that the affected nuclei were often hypertrophied. He considered that these inclusions were possibly caused by a filterable virus. Andrew et al., however, ascribed them to metabolic disturbances attendant upon senescence, since they appeared in the livers of senile human beings and aged mice. Both reports agreed that these bodies may be hypertrophied nucleoli. In the present experiment, the bodies developed in enlarged cells in focal areas in the liver after treatment with a carcinogenic substance. Andrew et al. also observed an increasing variation in liver-cell size and the development of very large liver cells in advancing age. This condition, as well as the nuclear inclusions, has been accentuated in the livers of mice used in this experiment. As in the previous experiment, a focal increase in reticulum in the liver was observed, and thick reticulum strands lay between the hypertrophied cells, while only a few delicate fibers were found in the areas of small cells (fig. 3).

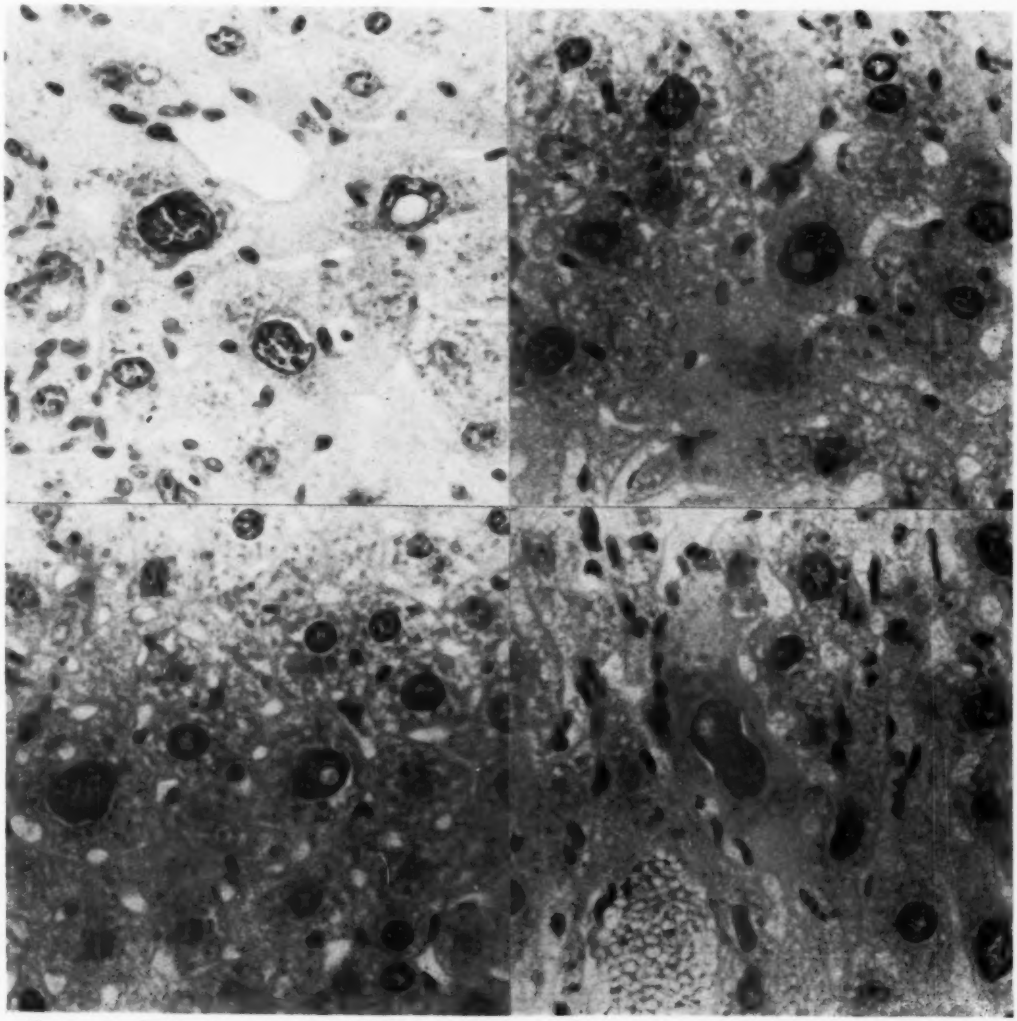

FIGURE 2.—Enlarged liver cells showing nuclear inclusions. Hematoxylin and eosin stain. × 560.

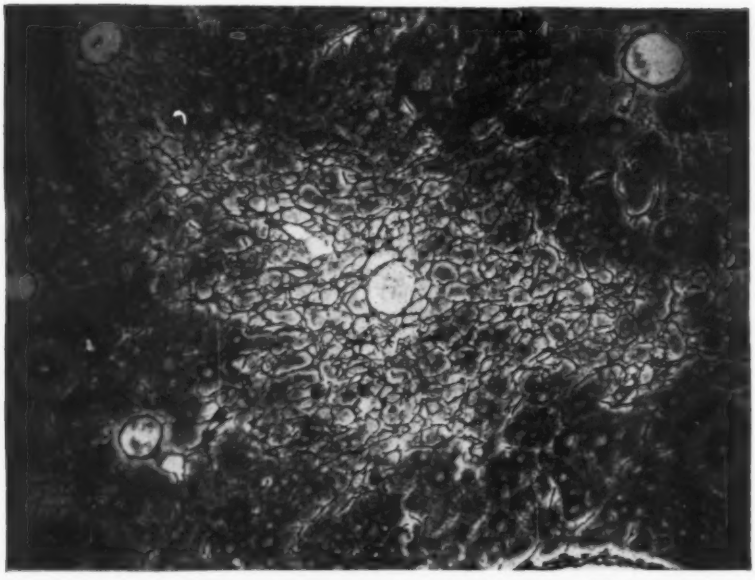

FIGURE 3.—Reticulum increase in area of large cells about central vein. Wilder reticulum stain. × 120.

TABLE 3.—*Induction of macroscopic hepatic changes in intact and castrate strain C mice and C mice castrated and implanted with testosterone propionate pellets following subcutaneous administration of 40 mg. of o-aminoazotoluene*

[Numerator = Mice killed
Denominator = Mice with hepatic changes]

Group	Number of mice	Time after first injection in months					Total	Incidence
		9	10	11	12	13		
								Percent
Intact females	37	8/5	4/4	4/4	4/4	17/17	37/34	92
Castrate females	47	8/7	4/1	4/4	7/6	24/14	47/32	68
Castrate females plus pellet	41	8/1	5/0	3/0	7/2	18/0	41/3	7
Intact males	46	3/0	3/0	5/0	3/0	32/0	46/0	0
Castrate males	40	3/3	1/1	5/4	4/3	27/27	40/38	95
Castrate males plus pellet	43	3/0	4/0	7/0	1/0	28/2	43/2	5

Castration exerted a definite influence upon the susceptibility of males by raising the incidence of hepatic changes from 0 percent in the intact animals to 95 percent in castrate litter mates. It is also indicated that castration increased the resistance of the females to the activity of the carcinogen.

The presence of testosterone propionate pellets in castrates of both sexes had a pronounced influence upon the induction of hepatic changes. The females showed an incidence of only 7 percent and the males an incidence of 5 percent. This result was clear-cut.

The occurrence of hepatomas is shown in table 4. It is seen that castrate males approached their intact female litter mates in susceptibility. Castrate females, however, revealed an incidence of 38 percent whereas their intact litter mates showed an incidence of 75 percent. Furthermore, the castrates had an average of two hepatomas and the intact females an average of four hepatomas. These observations emphasize the results suggested in tables 1 and 2, namely, that castration increased the resistance of the females. Furthermore few hepatomas arose in the castrate-plus-pellet group of either sex; none in the males and in only 7 percent of the females. These findings are significant.

TABLE 4.—*Induction of pulmonary tumors and hepatic tumors in intact and castrate strain C mice and C mice castrated and implanted with testosterone propionate pellets following subcutaneous administration of 40 mg. of o-aminoazotoluene*

[Numerator = Mice with pulmonary tumors
Denominator = Mice with hepatic tumors]

Group	Number of mice	Time after first injection in months [1]					Total	Incidence
		9	10	11	12	13		
								Percent
Intact females	37	5/2	1/4	3/2	3/4	15/16	27/28	73/75
Castrate females	47	3/0	1/1	3/1	6/6	19/10	32/18	68/38
Castrate females plus pellet	41	4/0	2/1	.....	6/2	8/0	20/3	49/7
Intact males	46	.....	1/0	5/0	1/0	18/0	25/0	54/0
Castrate males	40	0/2	.....	2/3	3/4	23/20	28/29	70/72
Castrate males plus pellet	43	1/0	1/0	3/0	1/0	19/0	25/0	54/0

[1] See table 3 for number of mice autopsied each month.

Table 4 also includes the occurrence of gross primary pulmonary tumors. It is seen that 40 mg. of the compound induced this type of tumor in all groups of mice, but the differences between groups are not considered to be significant.

DISCUSSION

The outcome of these studies confirmed the sex difference in susceptibility of strain C mice to induced hepatic lesions following administration of *o*-aminoazotoluene. They also showed that castration of males increased their susceptibility and castration of females increased their resistance and thus suggested that sex hormones may be at least partially responsible for the variation in susceptibility. The increased resistance of castrate females suggested that androgens may be responsible for the sex variation, because as Parkes (*8*) stated, "There is good experimental evidence that the adrenal of the female may show androgenic activity."

An experiment designed to test this possibility revealed that the administration of testosterone propionate to castrate males and females led to a remarkable decrease in their degree of susceptibility.

How the androgen influenced the susceptibility of the animals is not known. Parfentjev and Perlzweig (*9*) found that the urine of male mice contains a larger amount of proteins than does that of the females. Wicks (*10*) reported that this proteinurea is found almost exclusively in males and that castration of males reduced their protein excretion. Perhaps the androgenic influence enabled the mice to excrete more of the carcinogen or its derivatives.

Selye (*11*) observed an increase in kidney weight as well as hypertrophy of the epithelium of the convoluted tubules and the epithelium lining Bowman's capsule in testosterone-treated female mice. Crabtree (*12,13*) discovered that Bowman's capsule in female mice is composed of squamous cells whereas in males it is composed mostly of cuboidal cells. She found also (*14*) that in castrate males the capsule resembles that of intact females, and injection of testosterone to castrate males returned the structure of the capsules to that found in intact males.

Eschenbrenner (*15*) observed that oral administrations of chloroform produced necrosis of the convoluted tubules of the kidneys in male mice but not in females. Hepatomas were induced in females only, because the males died from renal damage when given a dose large enough to elicit liver necrosis. He later showed (*16*) that chloroform caused kidney necrosis in intact and testosterone-treated castrate males but failed to do so in intact females and castrate males.

Perhaps in the experiment reported here, the androgen produced a change in renal physiology which enabled the mice to excrete the carcinogen or its derivatives and thereby lessened its carcinogenic effects.

It would be of interest to ascertain the influence of testosterone treatment upon the incidence of spontaneous hepatomas in strain C mice, but these animals develop few such tumors. Strain C3H mice, however, are relatively susceptible to the development of spontaneous hepatomas (*17*), and in contrast with the sex variation in response to *o*-aminoazotoluene, the males are considerably more susceptible than their female litter mates (*18, 19*). The question arises whether, in contrast with the studies on induced hepatomas, testosterone propionate would not exert an inhibitory influence upon the development of spontaneous hepatomas.

Burns and Schenken (*20*) found fewer tumors in nonbreeding than in breeding strain C3H mice of both sexes but stated that the number of animals involved in their studies was too small to permit definite conclusions.

Schenken and Burns (*21*) administered testosterone propionate to 24 nonbreeding males and 24 nonbreeding females of strain C3H. None of the males and only 1 female developed a hepatoma. They also treated 59 nonbreeding males and 10 nonbreeding females with α-estradiol benzoate, and 15 males but no females developed hepatomas. Of 30 nonbreeding males and 9 nonbreeding females given injections of ketohydroxyestrin, 5 males and no females developed hepatomas. The authors did not evaluate the results of testosterone propionate injections but interpreted their findings as suggesting that estrogen treatment elevated the incidence of hepatomas in the males and depressed the incidence in females. They state that "the evaluation of the effect of estrogen treatment on the incidence of liver tumors must be considered as tentative, because the number of mice in many groups was small."

Shimkin and Wyman (*22*) presented data which they interpreted as suggesting that estrogenation of strain C3H males resulted in a lowered incidence of spontaneous hepatomas. They stated that untreated normal controls were not maintained, and further experimentation is essential before any conclusions can be made.

In view of the striking effect of testosterone treatment upon the induction of hepatomas by *o*-aminoazotoluene, further studies on the influence of sex hormones on the occurrence of spontaneous hepatomas in mice should be of interest.

Murphy's investigations (*23*) dealing with the effect of sex and testosterone on the incidence of spontaneous leukemia are also of considerable interest here. He used a strain of mice in which

females and males had an incidence of 88.4 and 53.5 percent, respectively. Castration resulted in an incidence of 90.3 in females and 97 percent in males. Ovariectomized females treated with testosterone propionate revealed an incidence of 58.3 percent. Thus, Murphy's observations with this leukemic strain are similar to those reported here with induced hepatic lesions in strain C mice.

The reader is referred to Furth's review (*24*) for further information concerning the influence of sex and sex hormones upon the development of leukemia in mice.

The occurrence of hemangio-endotheliomas in mice following application of *o*-aminoazotoluene is receiving more consideration. This type of tumor appeared in mice of strains A, C3H, C, and C57 black following administration of the crystalline compound (*2*) as well as in strain C mice following injections of olive-oil solutions (*25*), or the ingestion of a diet containing the dye (*26*). The spontaneous occurrence of this tumor in strain C animals is not known precisely, but the incidence is less than 5 percent. An interesting feature of the induced tumor is the variety of sites of origin. To summarize the sites of origin, the results obtained to date with strain C animals are presented in table 5, which includes previous experiments as well as the two recorded here.

TABLE 5.—*Summary of the sites of origin of hemangio-endotheliomas induced in strain C mice with o-aminoazotoluene*

Experiment No. or reference	Route of administration	Condition of azo dye	Amount administered	Number of mice	Sex	Distribution of hemangio-endotheliomas according to site of origin								Total
						Interscapular fat	Lung	Peritoneal fat	Subcutaneous tissue	Ovary	Adrenal cortex	Muscle	Spleen	Liver	
			Mg.			*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*
1	Subcutaneous at base of tail.	Crystalline	70	45	F	9	23		2			1			35
1	do	do	70	49	M	6	15								21
2	Subcutaneous in right axilla.	do	40	125	F	7		2	1				1		11
2	do	do	40	129	M	1		1	4				1	1	8
(2)	do	do	100	12	F	8	1	1	2	1	1				14
(2)	do	do	100	12	M	2			2						4
(27)	do	Dissolved in olive oil.	45	20	F	3				1					4
(27)	do	do	45	20	M	1			1						2
(28)	Oral	Incorporated in diet.	100–165	31	F	1	16	1							18
(28)	do	do	130–220	29	M		4								4

As stated before, the crystalline compound was injected subcutaneously at the base of the tail in the first experiment. Of 7 intact and 38 castrate females, 5 and 24, respectively, developed hemangio-endotheliomas. Of these mice, 1 and 8, respectively, had 2 such tumors. Of 7 intact and 42 castrate males, 2 and 17, respectively, developed this tumor. Of these male mice, 0 and 2, respectively, had 2 such tumors.

As shown in table 5, many of these tumors arose in the lungs. Similar findings attended an experiment in which the compound was incorporated in the diet; but few tumors arose in the lungs when the crystalline material or olive-oil solutions were given subcutaneously in the right axillary region. It is not known why the compound induced more hemangio-endotheliomas of the lung when placed subcutaneously at the base of the tail than in the axillary region.

The data in table 5 also show that females of strain C are more susceptible than males to the induction of hemangio-endothelioma with *o*-aminoazotoluene. In the second experiment, the mice received a total of 40 mg. of the crystalline compound subcutaneously in the right axillary region. Of the females, 37 intact, 47 castrate, and 41 castrated and given testosterone treatment, 4, 5, and 2, respectively, developed hemangio-endotheliomas. Of the males, 46 intact, 40 castrate, and 43 castrated and given testosterone treatment, 3, 5, and 0, respectively developed this tumor. These results, together with those of the first experiment and the other data in the table, suggest that sex susceptibility of strain C mice to induced hemangio-endotheliomas parallels their susceptibility to induced hepatic lesions and hepatomas.

By combining the results of the experiments reported here, it is found that of a total of 53 intact males 5 developed hemangio-endotheliomas, while similar tumors arose in 22 of 82 castrate males. This suggests that gonadectomy also increased the susceptibility of the males to induced hemangio-endotheliomas. The occurrence of this type of tumor in only 2 of 41 castrate females and in none of 43 castrate males bearing implanted pellets of testosterone suggests that the androgen increased their resistence. Thus, the effects of gonadectomy and testosterone upon the induction of hemangio-endotheliomas in strain C mice also parallel the effects of similar measures upon susceptibility to induced hepatic changes and hepatomas.

This similarity between the production of *o*-aminoazotoluene-induced hemangio-endotheliomas and hepatomas in strain C mice in respect to

sex variation in susceptibility and the influence of sex hormones upon susceptibility suggests that the same factors (metabolic or excretory) determined the induction of both types of tumors in these animals. These factors, however, did not exert any influence upon the production of pulmonary tumors in this strain.

SUMMARY

The influence of sex and sex hormones upon the susceptibility of strain C mice to hepatic lesions, hepatomas, pulmonary tumors, and hemangio-endotheliomas induced with *o*-aminoazotoluene was studied.

So far as hepatic lesions and hepatomas were concerned, intact females were far more susceptible than intact males; intact females were more susceptible than castrate females; castrate males approached intact females in their degree of susceptibility; castrate females and castrate males bearing testosterone propionate-cholesterol pellets approached intact males in their degree of susceptibility.

Intact females were more susceptible to hemangio-endotheliomas than were intact males. Castrate males were more susceptible to this tumor than were intact males. Few hemangio-endotheliomas arose in castrates of either sex bearing testosterone propionate-cholesterol pellets.

Administration of the carcinogen subcutaneously at the base of the tail induced many hemangio-endotheliomas in the lungs.

So far as pulmonary tumors were concerned, there was no significant difference in susceptibility between the sexes or between sex-hormone-treated groups of mice.

REFERENCES

(*1*) ANDERVONT, H. B., and EDWARDS, J. E.: Hepatic changes and subcutaneous and pulmonary tumors induced by subcutaneous injection of 3,4,5,6-dibenzcarbazole. J. Nat. Cancer Inst. 2: 139–149 (1941).

(*2*) ANDERVONT, H. B., GRADY, H. G., and EDWARDS, J. E.: Induction of hepatic lesions, hepatomas, pulmonary tumors, and hemangio-endotheliomas in mice with *o*-aminoazotoluene. J. Nat. Cancer Inst. 3: 131–153 (1942).

(*3*) HESTON, W. E.: Genetics of mammary tumors in mice. *In* A Symposium on Mammary Tumors in Mice. A. A. A. S. Publ. 22. Washington, D. C. (1945).

(*4*) ANDERVONT, H. B.: The susceptibility of five inbred strains of mice to liver changes induced by subcutaneous injection of 2-amino-5-azotoluene. Pub. Health Rep. 54: 1986–1991 (1939).

(*5*) SHIMKIN, M. B., and WHITE, J.: Absorption rate of hormone-cholesterol pellets. Endocrinology 29: 1020–1025 (1941).

(*6*) FINDLAY, G. M.: Intranuclear bodies in cells of mice. Brit. J. Exper. Biol. 13: 223–229 (1932).

(*7*) ANDREW, W., BROWN, H. M., and JOHNSON, J. B.: Senile changes in the liver of mouse and man, with special reference to the similarity of the nuclear alterations. Am. J. Anat. 72: 199–221 (1943).

(*8*) PARKES, A. S.: The adrenal-gonad relationship. Physiol. Rev. 25: 203–254 (1945).

(*9*) PARFENTJEV, I. A., and PERLZWEIG, W. A.: The composition of the urine of white mice. J. Biol. Chem. 100: 551–555 (1933).

(*10*) WICKS, L. F.: Sex and proteinuria of mice. Proc. Soc. Exper. Biol. & Med. 48: 395–400 (1941).

(*11*) SELYE, H.: The effect of testosterone on the kidney. J. Urol. 42: 637–641 (1939).

(*12*) CRABTREE, C.: Sex differences in the structure of Bowman's capsule in the mouse. Science 91: 299 (1940).

(*13*) ———: The structure of Bowman's capsule as an index of age and sex variations in normal mice. Anat. Rec. 79: 395–413 (1941).

(*14*) ———: Structure of Bowman's capsule in castrate and testosterone treated male mice as index of hormonal effects on renal cortex. Endocrinology 29: 197–203 (1941).

(*15*) ESCHENBRENNER, A. E.: Induction of hepatomas in mice by repeated oral administration of chloroform, with observations on sex differences. J. Nat. Cancer Inst. 5: 251–255 (1945).

(*16*) ———: Sex differences in kidney morphology and chloroform necrosis. Science 102: 302–303 (1945).

(*17*) ANDERVONT, H. B., and MCELENEY, W. J.: Spontaneous tumors in a subline of strain C3H mice. J. Nat. Cancer Inst. 1: 737–744 (1941).

(*18*) ANDERVONT, H. B.: The occurrence of spontaneous and induced pulmonary and liver tumors in strain C3H mice. Pub. Health Rep. 54: 1158–1169 (1939).

(*19*) BURNS, E. L., and SCHENKEN, J. R.: Spontaneous primary hepatomas in mice of strain C3H. A study of incidence, sex distribution and morbid anatomy. Am. J. Cancer 39: 25–35 (1940).

(*20*) BURNS, E. L., and SCHENKEN, J. R.: Spontaneous primary hepatomas in mice of strain C3H. II. The influence of breeding on their incidence. Cancer Research 3: 691–692 (1943).

(*21*) SCHENKEN, J. R., and BURNS, E. L.: Spontaneous primary hepatomas in mice of strain C3H. III. The effect of estrogens and testosterone proprionate on their incidence. Cancer Research 3: 693–696 (1943).

(*22*) SHIMKIN, M. B., and WYMAN, R. S.: Mammary tumors in male mice implanted with estrogen-cholesterol pellets. J. Nat. Cancer Inst. 7: 71–75 (1946).

(*23*) MURPHY, J. B.: The effect of castration, theelin and testosterone on the incidence of leukemia in a Rockefeller Institute strain of mice. Cancer Research 4: 622–624 (1944).

(*24*) FURTH, J.: Recent experimental studies on leukemia. Phys. Rev. 26: 47–76 (1946).

(*25*) ANDERVONT, H. B., and EDWARDS, J. E.: Carcinogenic action of two azo compounds in mice. J. Nat. Cancer Inst. 3: 349–354 (1943).

(*26*) ANDERVONT, H. B., and WHITE, J.: Effect of two azo compounds when added to the diet of mice. J. Nat. Cancer Inst. 4: 583–586 (1944).

RELATIONSHIP BETWEEN THE LETHAL YELLOW (A^y) GENE OF THE MOUSE AND SUSCEPTIBILITY TO SPONTANEOUS PULMONARY TUMORS[1,2]

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INTRODUCTION

In previous papers (*1*, *2*), it was reported that susceptibility to induced pulmonary tumors in mice is associated with a number of unit characters, among which is the character lethal yellow. The yellow F_1 hybrid mice from a cross between strain A and strain Y were more susceptible to pulmonary tumors induced by intravenous injection of 20-methylcholanthrene than were their brown litter mates.

The next step, after the relationship of lethal yellow to induced pulmonary tumors was established, was to ascertain whether or not the gene was likewise associated with spontaneous pulmonary tumors. Data presented herein show that it is.

EXPERIMENTAL PROCEDURE AND RESULTS

Strain A and strain Y, the strains used in the induced-tumor experiment, were used as the parent strains. Both strains were of the colonies maintained at the National Cancer Institute, although both were originally obtained from the Roscoe B. Jackson Memorial Laboratory, strain A in 1938 and strain Y in 1936.

Strain A is a well-known, high-pulmonary-tumor strain. Incidences of 53, 79, and 90 percent have been reported for mice of our colony at 12, 15, and 18 months of age, respectively (*3*). The strain can be considered homozygous for practically all genetic factors since brother-sister inbred generations now total over 70.

Strain Y, with which the A^y gene was introduced, has a medium degree of susceptibility to pulmonary tumors. Andervont (*4*) reported an incidence of from 10 to 20 percent of spontaneous pulmonary tumors in this strain. The animals used in these experiments were of the twenty-fifth generation of brother-sister inbreeding subsequent to the time that the strain was obtained from the Jackson Memorial Laboratory, and the stock had been inbred prior to that time. Since the effect of the gene is lethal when homozygous (*5–7*), yellow animals are always heterozygous for one of the other genes of the allelic series. In this strain it is the non-agouti (a) gene. Thus, in maintaining the strain, although yellow animals are selected for breeders, one-third of their offspring are homozygous, non-agouti, and brown in color since this subline also carries brown. Despite the fact that the yellow animals are heterozygous at this locus, one can consider that the inbreeding would have resulted in a strain homozygous for practically all genes on all chromosomes other than the pair marked by the agouti locus. Furthermore, owing to crossing over, practically all genes on this chromosome, with the possible exception of any closely linked to agouti, also would be expected to be homozygous.

The relationship of the A^y gene to spontaneous-pulmonary-tumor development was observed in the F_1 hybrids resulting from crossing strain A females to strain Y males. One could expect that such hybrids would be genetically uniform except that one-half would be heterozygous ($A^y a$) and yellow in color, and the other half would be brown ($a\ a$). The situation thus would offer evidence that deviation in tumor incidence of the yellow mice from that of the brown could be attributed to the presence of the A^y gene.

Two lots of the F_1 hybrids were produced, with a time interval of approximately 2 years. In the first lot 111 hybrids were weaned and set aside between June and October 1943 to be killed and observed for pulmonary tumors at 15 months of age. Of these, 82 lived to 15 months of age and were autopsied as scheduled. The tumor incidence for this first lot was determined on these 82 animals, since it is essential to control the age factor in comparing pulmonary-tumor incidences; furthermore, some of those which died earlier had pneumonia, preventing an accurate determination of whether or not lung nodules were present. It is assumed that the factors causing the death of the animals dying earlier than 15 months of age were unrelated to pulmonary-tumor development. Diagnosis of the tumors was made with the aid of the dissecting microscope and confirmed with histologic sections.

The tumor incidences for the 2 color groups are given in table 1. Since no sex difference has been observed in regard to spontaneous pulmonary tumors, the lots are not segregated as to sex. Of

TABLE 1.—*Pulmonary tumor development in AYF$_1$ hybrids 15 months of age*

Experiment	Yellow ($A^y a$)		Brown ($a\ a$)	
	Tumor	Nontumor	Tumor	Nontumor
	Number	*Number*	*Number*	*Number*
1	16	22	9	35
2	15	23	10	35
Total	31	45	19	70

[1] Received for publication April 15, 1947.
[2] Presented at the annual meeting of the American Association for Cancer Research, Chicago, May 16–17, 1947.

the 82 mice, 38 were yellow ($A^y a$), and 44 were brown ($a\,a$); of the 38 yellows, 16 had pulmonary tumors while such tumors were found in only 9 of the 44 browns. While these results suggested that the yellow mice had a higher degree of susceptibility to pulmonary tumors than did their brown siblings, the difference between the two groups was not statistically significant.

It was thus essential to increase the numbers by adding a second lot of F_1 hybrids from strain A females and strain Y males. Accordingly 153 were weaned and set aside between January and May 1945. Of this lot 83 were autopsied at the age of 15 months and were employed in ascertaining the tumor incidence. They included 38 yellows ($A^y a$), of which 15 had pulmonary tumors, and 45 browns ($a\,a$), of which 10 had pulmonary tumors. Again the difference between the pulmonary-tumor incidences of the 2 color groups was not statistically significant, but it was, when the data of the first and second lot were combined, making a total of 165 animals. Thirty-one of the seventy-six yellow mice had tumors, whereas only 19 of the 89 browns had tumors. This difference is highly significant; $\chi^2=7.425$; $P<0.01$.

Since the A^y gene is known to have an influence on body size, as described by Danforth (*8*), weights were taken on the second lot at 6 and 12 months of age and again when the mice were autopsied at 15 months. The average weights of the yellow mice as contrasted with those of the brown sibs are given in table 2. The effect of the lethal yellow gene on weight was particularly apparent at 6 months, for at that age the yellow mice were much heavier than their brown sibs. As the animals aged, the yellow mice lost weight, so that at 12 months the difference was not so great, and at 15 months there was no difference between the average weights of the two color groups.

TABLE 2.—*Average weights of the AYF$_1$ hybrids*

Group	Sex	Number	Average weights at—		
			6 months	12 months	15 months
			Grams	*Grams*	*Grams*
Yellow ($A^y a$)	M	9	48.56	36.22	30.38
	F	29	46.55	35.83	24.07
Brown ($a\,a$)	M	18	34.56	30.00	28.94
	F	27	26.93	24.81	24.70

DISCUSSION

The development of pulmonary tumor in the mouse is to be considered as under the control of multiple genetic and nongenetic factors. The tumor appears when the cumulative effect of both genetic and nongenetic factors surpass a physiologic threshold. In this general concept a carcinogen is to be viewed as a potent nongenetic factor, the introduction of which greatly increases the probability that this threshold will be surpassed at a much earlier age.

Considered in this way, the carcinogens have proved to be a convenient tool in the genetic analysis of pulmonary tumors since by their injection the whole picture of tumor development is shifted to an earlier age with a greater number of nodules; thus the genetic differences can be much more readily detected. By this means, relationships between induced pulmonary tumors and the several known genes, including flexed tail, shaker-2, and lethal yellow were demonstrated (*1, 2*). It was not indicated whether the relationship to the first two genes was true linkage or an effect of the gene tested. In the case of the lethal yellow gene, it was evident that the increase in susceptibility to the induced pulmonary tumors was an added effect of the lethal yellow gene.

According to the foregoing concept, one could expect this relationship between pulmonary tumors and these known genes to hold for spontaneous pulmonary tumors as well as for those induced with the carcinogens, although it would be more difficult to demonstrate. Data presented herein bear out this expectation in showing that the lethal yellow gene does have an effect on the occurrence of the spontaneous tumors. It is planned to make similar tests in regard to the other two genes.

In a previous paper (*2*), it was suggested that the physiologic processes through which the lethal yellow gene increases body weight may also be responsible for the increase in susceptibility to induced pulmonary tumors. The same suggestion would likewise apply to the effect on spontaneous pulmonary tumors. Supporting evidence was cited in reports indicating a general increase in liability to cancer in overweight individuals and in the work of Tannenbaum (*9–11*) indicating that dietary restriction may decrease susceptibility to a number of types of tumors in mice, pulmonary tumors included. Recently Larsen and Heston (*12*) reported that certain diets decrease susceptibility to pulmonary tumors in mice and interpreted the effect as one which might generally be observed in animals fed deficient diets. In line with these findings, Dickerson and Gowen (*13*) from their recent analysis of the obesity effect of the lethal yellow gene concluded that obesity is the result of increased appetite with reduced energy requirements.

In the mice of the present experiment the tumors were observed when the animals were autopsied at 15 months of age, and at this time the yellow mice had lost their obesity. Despite this fact, however, the physiologic action of the lethal yellow gene which results in obesity may be the same as that which results in increased susceptibility to pulmonary tumors. These tumors grow slowly, and

many of them probably arose several months prior to the time of autopsy. On the other hand, there is no reason to assume that the physiologic action increasing susceptibility could not occur long before the tumors appear. It is also conceivable that the same genic action which earlier resulted in obesity might continue to influence tumor susceptibility, especially if the obesity is lost owing to the insertion of another factor into the physiologic complex, and this appears to be the case. These yellow hybrids probably lost their obesity because of the onset of amyloidosis and its accompanying renal disease, a disorder that occurs in both strain A and strain Y mice. All the F_1 hybrids showed both amyloidosis and the renal disease at the time of autopsy.

Dickie and Woolley (*14*) recently reported that in their F_1 hybrids, resulting from mating their strain of yellow mice to the *cc* strain, the yellows likewise did not hold their increased weight throughout the life span. In their hybrids, however, the maximum difference was maintained from about the seventh to the eighteenth month of age, after which the yellow mice began to lose weight. By the twenty-fourth month, they were not significantly heavier than their nonyellow siblings. No specific suggestion why their yellow mice lost their obesity was offered.

SUMMARY

The presence of the lethal yellow (A^y) gene increases the incidence of spontaneous pulmonary tumors in mice, as demonstrated by the higher incidence in the yellow ($A^y a$) F_1 hybrids from the cross between strain A and strain Y than in their brown ($a\ a$) litter mates. This effect is similar to that previously demonstrated for induced pulmonary tumors.

REFERENCES

(*1*) Heston, W. E.: Relationship between susceptibility to induced pulmonary tumors and certain known genes in mice. J. Nat. Cancer Inst. 2: 127–132 (1941).

(*2*) ———: Relationship between the lethal yellow (A^y) gene of the mouse and susceptibility to induced pulmonary tumors. J. Nat. Cancer Inst. 3: 303–308 (1942).

(*3*) ———: Inheritance of susceptibility to spontaneous pulmonary tumors in mice. J. Nat. Cancer Inst. 3: 79–82 (1942).

(*4*) Andervont, H. B.: Susceptibility of mice to spontaneous, induced, and transplantable tumors; comparative study of 8 strains. Pub. Health Rep. 53: 1647–1665 (1938).

(*5*) Kirkham, W. B.: Embryology of the yellow mouse. Anat. Rec. 11: 480–481 (1917).

(*6*) Little, C. C.: A note on the fate of individuals homozygous for certain color factors in mice. Am. Nat. 53: 185–187 (1919).

(*7*) Robertson, G. G.: An analysis of the development of homozygous yellow mouse embryos. J. Exper. Zool. 89: 197–227 (1942).

(*8*) Danforth, C. H.: Hereditary adiposity in mice. J. Hered. 18: 153–162 (1927).

(*9*) Tannenbaum, A.: Relationship of body weight to cancer incidence. Arch. Path. 30: 509–517 (1940).

(*10*) ———: The genesis and growth of tumors. II. Effects of caloric restriction per se. Cancer Research 2: 460–467 (1942).

(*11*) ———: The genesis and growth of tumors. III. Effects of a high fat diet. Cancer Research 2: 468–475 (1942).

(*12*) Larsen, C. D., and Heston, W. E.: Effects of cystine and calorie restriction on the incidence of spontaneous pulmonary tumors in strain A mice. J. Nat. Cancer Inst. 6: 31–40 (1945).

(*13*) Dickerson, G. E., and Gowen, J. W.: Hereditary obesity and efficient food utilization in mice. Science 105: 496–498 (1947).

(*14*) Dickie, M. M., and Woolley, G. W.: The age factor in weight of yellow mice. Weight reduction of aging yellows and "thin-yellows" revealed in littermate comparisons. J. Hered. 37: 365–368 (1946).

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